

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61L 24/00, C08B 37/08, A61L 27/00	A1	(11) International Publication Number: WO 00/16818 (43) International Publication Date: 30 March 2000 (30.03.00)
(21) International Application Number: PCT/EP99/06913 (22) International Filing Date: 17 September 1999 (17.09.99) (30) Priority Data: 09/156,829 18 September 1998 (18.09.98) US (71)(72) Applicants and Inventors: AESCHLIMANN, Daniel [CH/GB]; Biochemical Sciences Building, Cardiff University, Museum Avenue, P.O. Box 991, Cardiff, CF1 3US (GB). BULPITT, Paul [GB/GB]; 24 Cotswold Close, Basingstoke, Hampshire (GB). (74) Agent: VON MENGES, Albrecht; Uexküll & Stolberg, Beselerstrasse 4, D-22607 Hamburg (DE).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: FUNCTIONALIZED DERIVATIVES OF HYALURONIC ACID AND FORMATION OF HYDROGELS IN SITU USING SAME (57) Abstract Methods for chemical modification of hyaluronic acid, formation of amine or aldehyde functionalized hyaluronic acid, and the cross-linking thereof to form hydrogels are provided. Functionalized hyaluronic acid hydrogels of this invention can be polymerized <i>in situ</i> , are biodegradable, and can serve as a tissue adhesive, a tissue separator, a drug delivery system, a matrix for cell cultures, and a temporary scaffold for tissue regeneration.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

FUNCTIONALIZED DERIVATIVES OF HYALURONIC ACID AND FORMATION OF HYDROGELS IN SITU USING SAME

5

TECHNICAL FIELD OF THE INVENTION

This invention is directed to biomaterials for spatially and temporally controlled delivery of bioactive agents such as drugs, growth factors, cytokines or cells. In particular, this invention teaches versatile methods for chemical crosslinking of high molecular weight hyaluronic acid under physiological conditions *in situ*, to form polymerizable biodegradable materials. The methods are based on the introduction of functional groups into hyaluronic acid (HA) via formation of an active ester at the carboxylate of the glucuronic acid moiety as an intermediate and subsequent substitution with a side chain containing a nucleophilic group on one end and a (protected) functional group on the other end. The introduced functional groups allow for crosslinking of the HA derivatives. Crosslinked hyaluronic acid hydrogels of this invention are useful in various surgical applications and as a temporary scaffold for tissue regeneration, e.g., in cartilage repair.

20

Repair of articular cartilage

BACKGROUND OF THE INVENTION

The failure of regenerating persistent hyaline cartilage by surgical procedures prompted investigators to attempt repair using biological strategies.

- 2 -

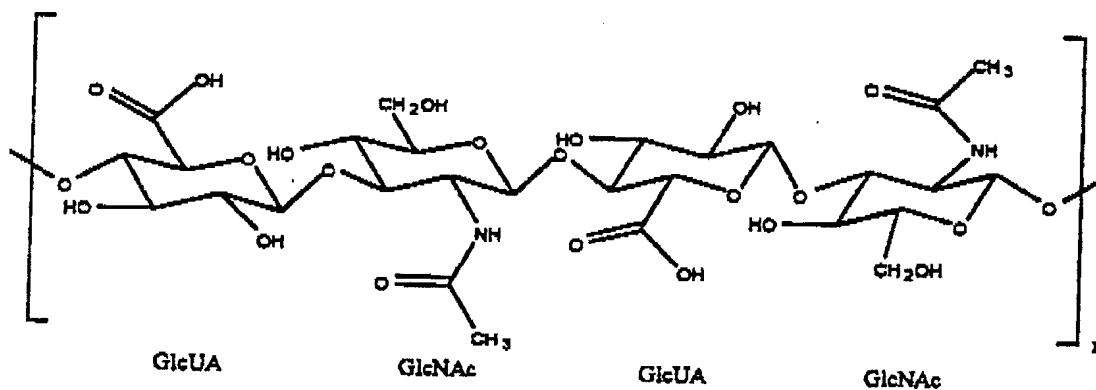
The biological repair of articular cartilage is, with a few exceptions, still at an experimental stage. Biological cartilage repair has been approached in two basic ways. First, autologous chondrocytes have been transplanted into a lesion to induce repair (Grande et al., J. Orthop. Res. 7, 208-214 (1989); Brittberg et al., New Engl. J. Med. 331, 889-895 (1994); Shortkroff et al., Biomaterials 17, 147-154 (1996)). Chondrocytes may be obtained from a low-loaded area of a joint and proliferated in culture (see Grande; Brittberg; Shortkroff, supra), or mesenchymal stem cells may be harvested, e.g., from the iliac crest marrow, and induced to differentiate along the chondrocyte lineage using growth factors (Harada et al., Bone 9, 177-183 (1988); Wakitani et al., J. Bone Joint Surg. 76-A, 579-592 (1994)). The chondrocyte transplantation procedures currently attempted clinically, although promising, are hampered because technically they are very challenging, the cell preparation is very expensive, and the potential patient pool is limited by age, defect location, history of disease, etc. Cells have also been transplanted into cartilage defects in the form of perichondral grafts, e.g. obtained from costal cartilage, but with limited success due to the limit in donor material and the complication of endochondral ossification of the graft site observed in longterm follow-up (Amiel et al., Connect. Tissue Res. 18, 27-39 (1988); O'Driscoll et al., J. Bone Joint Surg. 70-A, 595-606 (1988); Homminga et al., Acta Orthop. Scand. 326-329 (1989); Homminga et al., J. Bone Joint Surg. 72-B, 1003-1007 (1990)). A second approach is aimed at the recruitment of mesenchymal stem cells from the surrounding connective tissue, e.g., synovium, using chemotactic and/or mitogenic factors (Hunziker and Rosenberg, J. Bone Joint Surg. 78-A, 721-733 (1996); see also U.S. Patent No. 5,368,858). The availability of growth factors and cytokines in recombinant form and the lack of complicated cell transplantation make this procedure a very attractive alternative. The shortcoming of both procedures is the difficulty to stably anchor the repair inducing factors, whether tissue grafts, cells or growth factors, within the defect site. Also, outlining of the space that is to be repaired, e.g., by filling it with a matrix material, appears to be crucial to recreate a level cartilage surface (Hunziker and Rosenberg, supra). Thus far, the availability

of candidate matrix materials has been the limiting factor, and anchoring of materials seeded with chondrocytes and/or chondrogenic factors difficult, explaining the unsatisfactory results obtained with currently available materials such as polylactic acid and polyglycolic acid scaffolds (Freed et al., J. Biomed. Mat. Res. 28, 891-899 (1994); Chu et al., J. Biomed. Mat. Res. 29, 1147-1154 (1995)), calcium phosphate minerals (Nakahara et al., Clin. Orthop. 276, 291-298 (1992)), fibrin sealants (Itay et al., Clin. Orthop. 220, 284-303 (1987)), and collagen gels (Wakitani et al., J. Bone Joint Surg. 71-B, 74-80 (1989)). We have developed novel biodegradable materials based on hyaluronic acid which are optimized for the biological requirements posed on a repair material in a synovial joint and allow *in situ* polymerization.

Biology of hyaluronic acid and its therapeutic use

Hyaluronic acid (HA) is unique among glycosaminoglycans in that it is not covalently bound to a polypeptide. HA is also unique in having a relatively simple structure of repeating nonsulfated disaccharide units composed of D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) (Scott et al., The Chemistry, Biology and Medical Applications of Hyaluronan and Its Derivatives, T.C. Laurent (ed.), Portland Press, London, (hereinafter "Hyaluronan and Its Derivatives"), pp. 7-15 (1998)). Its molecular mass is typically several million Daltons. HA is also referred to as hyaluronan or hyaluronate, and exists in several salt forms (see formula I).

(I)



- 4 -

HA is an abundant component of cartilage and plays a key structural role in the organization of the cartilage extracellular matrix as an organizing structure for the assembly of aggrecan, the large cartilage proteoglycan (Laurent and Fraser, FASEB J. 6, 2397-2404 (1992); Mörgelin et al., Biophys. Chem. 50, 113-128 (1994)). The noncovalent interactions of aggrecan and link protein with HA lead to the assembly of a large number of aggrecan molecules along the HA-chain and mediate retention of aggrecan in the tissue. The highly negatively charged aggrecan/HA assemblies are largely responsible for the viscoelastic properties of cartilage by immobilizing water molecules. A number of cell surface receptors for HA have been described and shown to play a critical role in the assembly of the pericellular matrix of chondrocytes and other cells, e.g., isoforms of CD44 and vertebrate homologues of Cdc37 (Knudson and Knudson, FASEB J. 7, 1233-1241 (1993); Grammatikakis et al., J. Biol. Chem. 270, 16198-16205 (1995)), or to be involved in receptor-mediated endocytosis and degradation of HA to control HA levels in tissues and body fluids (Laurent and Fraser, *supra*; Fraser et al., Hyaluronan and Its Derivatives, pp. 85-92 (1998)). Blocking of the interaction of these receptors with HA in prechondrogenic micromass cultures from embryonic limb bud mesoderm inhibits chondrogenesis, indicating that the establishment and maintenance of a differentiated chondrocyte phenotype is at least in part dependent on HA and HA-receptor interactions (Maleski and Knudson, Exp. Cell. Res. 225, 55-66 (1996)).

HA and its salts are currently being used in therapy for arthropathies by intraarticular injection (Stachnan et al., Ann. Rheum. Dis. 49, 949-952 (1990); Adams, Hyaluronan and Its Derivatives, pp. 243-253 (1998)), in ophthalmic surgery for intraocular lens implantation Denlinger, Hyaluronan and Its Derivatives, pp. 235-242 (1998), to promote wound healing in various tissues (King et al., Surgery 109, 76-84 (1991)), or more recently, in derivatized and/or crosslinked form to manufacture thin films which are used for tissue separation (for review see Laurent and Fraser, *supra*; Weiss, Hyaluronan and Its Derivatives, pp. 255-266 (1998); Larsen, Hyaluronan and Its Derivatives, pp. 267-281 (1998); Band, Hyaluronan and

- 5 -

Its Derivatives, pp. 33-42 (1998)). Extensive efforts have been made by various laboratories to produce derivatives of HA with unique properties for specific biomedical applications. Most of the developments have been focusing on the production of materials such as films or sponges for implantation and the substitution of HA with therapeutic agents for delayed release and/or prolonged effect (for review see Band, *supra*; Prestwich et al., Hyaluronan and Its Derivatives, pp. 43-65 (1998); Gustafson, Hyaluronan and Its Derivatives, pp. 291-304 (1998)). Strategies have included esterification of HA (U.S. Patent Nos. 4,957,744 and 5,336,767), acrylation of HA (U.S. Patent No. 5,410,016), and cross-linking of HA using divinyl sulfone (U.S. Patent No. 4,582,865) or glycidyl ether (U.S. Patent No. 4,713,448). However, the modified HA molecules show altered physical characteristics such as decreased solubility in water and/or the chemical reaction strategies used are not designed for crosslinking of HA under physiological conditions (in an aqueous environment, at pH 6.5-8.0).

It is well known that polyaldehydes can be generated by oxidizing sugars using periodate (Wong, CRC Press, Inc., Boca Rayton, FL, pp. 27 (1993); European Patent No. 9615888). Periodate treatment oxidizes the proximal hydroxyl groups (at C2 and C3 carbons of glucuronic acid moiety) to aldehydes thereby opening the sugar ring to form a linear chain (Scheme 1). While periodate oxidation allows for the formation of a large number of functional groups, the disadvantage is the loss of the native backbone structure. Consequently, the generated derivative may not be recognized as HA by cells. In fact hydrogels formed by using periodate oxidized HA as a crosslinker, e.g., in combination with the HA-amines described herein, showed very limited tissue transformation and poor cellular infiltration in the rat ectopic bone formation model (Fig. 6). This is in sharp contrast to the HA-aldehyde derivatives described herein.

The introduction of free amino groups on HA, which could be used for further convenient coupling reactions under mild physiological conditions, has been a subject of great interest. Previous methods have produced a free amino group on high molecular weight HA by alkaline *N*-deacylation of its glucosamine

- 6 -

moiety (Curvall et al., Carbohydr. Res. 41, 235-239 (1975); Dahl et al., Anal. Biochem. 175, 397-407 (1988)). However, concomitant degradations of HA via beta-elimination in the glucuronic acid moiety was observed under the harsh reaction conditions needed. This is of particular concern because low molecular weight HA fragments, in contrast to high molecular weight HA, have been shown to be capable of provoking inflammatory responses (Noble et al., Hyaluronan and Its Derivatives, pp. 219-225 (1998)). An early report claimed that carbodiimide-catalyzed reaction of HA with glycine methyl ester, a monofunctional amine, led to the formation of an amide linkage (Danishefsky and Siskovic, Carbohydr. Res. 16, 199-201 (1971)). This however, has been proven by a number of studies not to be the case (Kuo et al., Bioconjugate Chem. 2, 232-241 (1991); Ogamo et al., Carbohydr. Res. 105, 69-85 (1982)). Under mildly acidic conditions the unstable intermediate *O*-acylisourea is readily formed, which in the absence of nucleophiles, rearranges by a cyclic electronic displacement to a stable *N*-acylurea (Kurzer and Douraghi-Zedeh, Chem. Rev. 67, 107-152 (1967)). This *O*-*N* migration of the *O*-acylisourea also occurs when the nucleophile is a primary amine (Kuo et al., *supra*) and any amide formation that does occur is insignificant as reported by Ogamo et al., *supra*. Experiments where high molecular weight HA ($M_r \sim 2 \times 10^6$ Da) was reacted with an excess of the fluorescent label 5-aminofluorescein in the presence of the carbodiimide EDC achieved only 0.86% of theoretical labelling. The introduction of a terminal hydrazido group on HA with a variable spacer has recently been achieved and has led to the ability to conduct further coupling and crosslinking reactions (Pouyani and Prestwich, Bioconjugate Chem. 5, 339-347 (1994), U.S. Patent Nos. 5,616,568, 5,652,347, and 5,502,081; Vercruysse et al., Bioconjugate Chem. 8, 686-694 (1997))

It is the objective of this invention to provide a method for more versatile modification of HA with various functional groups that allow for crosslinking of the HA derivatives under physiological conditions. It is another objective that the method of functionalization does not compromise the molecular weight or chemical identity (except of the target carboxyl group for coupling) of

- 7 -

HA. It is a further objective that the method of functionalization provides HA molecules that are well tolerated *in vivo* and are biodegradable.

It is also the objective of this invention to identify HA derivatives and methodology for *in situ* polymerization thereof to provide a biodegradable scaffold
5 for tissue regeneration. It is another objective that the HA materials can be polymerized in the presence of cells to serve as a vehicle for cell transplantation. It is a further objective to provide methodology for functionalization and cross-linking of HA that allows for variations in the biomechanical properties of the formed gels as well as in the sensitivity to cellular infiltration and degradation.

10

SUMMARY OF THE INVENTION

Biomaterials for spatially and temporally controlled delivery of bioactive agents such as drugs, growth factors, cytokines or cells, are a key factor for tissue repair. In particular, *in situ* polymerizable biodegradable materials are needed for cartilage resurfacing that are designed to withstand the mechanical
15 forces in a joint. We have developed a versatile method for chemical crosslinking of high molecular weight hyaluronic acid under physiological conditions. The method is based on the introduction of functional groups into hyaluronic acid by formation of an active ester at the carboxylate of the glucuronic acid moiety and subsequent substitution with a side chain containing a nucleophilic group on one end and a
20 (protected) functional group on the other end. We have formed hyaluronic acid with amino or aldehyde functionality, and formed hydrogels with modified hyaluronic acid and bifunctional crosslinkers or mixtures of hyaluronic acid carrying different functionalities using active ester- or aldehyde-mediated reactions. Physical and chemical properties of the hydrogels of this invention were evaluated using
25 biomechanical testing, and by assaying sensitivity towards degradation by glycosidases such as testicular hyaluronidase. Biocompatibility was evaluated using cell culture assays and subcutaneous implantation of the hyaluronic acid materials in rats. This *in vivo* assay is also the established model for induction of ectopic bone formation by members of the transforming growth factor β family (TGF- β), and

- 8 -

several crosslinked hyaluronic acid materials of this invention gave excellent ectopic bone formation *in vivo* when loaded with appropriate growth factor(s).

As set forth below in the detailed description of the invention, the compositions of the invention have many therapeutic uses. For example,

5 compositions of the invention may be used to stem hemorrhage in general surgery, reconstruct nerves and vessels in reconstructive, neuro- and plastic surgery, and to anchor skin, vascular, or cartilage transplants or grafts in orthopedic, vascular, and plastic surgery. Compositions of the invention are also useful as vehicles for the delivery of cells or bioactive molecules such as growth factors to stimulate focal

10 repair. Local delivery of growth factors facilitates wound healing and tissue regeneration in many situations, not only in promoting bone formation and stimulating cartilage repair in orthopedic procedures, but also, e.g., in treating pathological wound conditions such as chronic ulcers. These compositions may also serve as a scaffold to generate artificial tissues through proliferation of

15 autologous cells in culture. On the other hand, the anti-adhesive property of some compositions with respect to cells render such compositions particularly suitable to generate tissue separations and to prevent adhesions following surgery. The viscoelastic properties of HA make it particularly well suited for this purpose, and it is used clinically to achieve temporal pain relief by repeated intraarticular injections

20 in arthropathies as a "joint lubricant", as a protective agent for eye irritations and in ophthalmic surgery, as a barrier to cells in facial and other reconstructions in plastic surgery and dentistry, in reconstructive surgery of tendons, in surgical procedures in the urogenital system, and in thoracic surgery. The injectable nature of the compositions of the invention also renders them suitable for tissue augmentation in

25 plastic surgery, where the HA matrix serves primarily as an inert biocompatible filler material (Balasz and Laurent, Hyaluronan and Its Derivatives, pp. 325-326 (1998)), e.g., for filling dermal creases or lip reconstruction.

HA hydrogels match several of the desired properties for a biodegradable material biocompatible with cells. The relatively simple repetitive

30 structure of HA allows for specific modification and introduction of a large number

- 9 -

of functional groups, for crosslinking to generate hydrogels with excellent physical properties. HA hydrogels have also successfully been used as a delivery vehicle in chondrocyte transplantation studies (Robinson et al., Calcif. Tissue Int. 46, 246-253 (1990)) and HA has proven its biocompatibility in various forms in clinical practice
5 (for review see Laurent and Fraser, *supra*; Balazs and Laurent, *supra*).

The reaction mechanisms we have explored for *in situ* polymerization of HA derivatives are compatible with an aqueous environment and are non-toxic to cells. The aldehyde-mediated crosslinking strategies follow reactions occurring physiologically in crosslinking of fibrillar collagens and elastin.
10 NHS-esters provide an alternative for rapid formation of stable bonds under physiological conditions, primarily by reaction with primary amines. The technology of NHS-ester-mediated protein crosslinking has been developed for materials with applications in plastic surgery that require *in situ* polymerization (U.S. Patent No. 5,413,791).

15 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the results of a ninhydrin test after reductive alkylation of HA and HA-aldehyde in the presence of putrescine. Reductive alkylation was carried out with an excess of putrescine in the presence of pyridine borane. HA or derivatives thereof were purified by repeated ethanol precipitation prior to detection
20 of free amino groups on the HA chain by using the ninhydrin test (Sheng et al., Anal. Biochem. 211, 242-249 (1993)).

Fig. 2 shows ¹H NMR of native HA (Fig. 2A) and an HA-derivative with protected aldehyde functionality (Fig 2B) in D₂O at 300 MHz. Peaks are assigned as indicated on the structural formula.

25 Fig. 3 shows ¹H NMR of HA-derivatives with amine functionality formed from putrescine (Fig. 3A), histidine (Fig. 3B), lysine (Fig. 3C), and adipic dihydrazide (Fig. 3D) in D₂O at 300Mhz. Peaks are assigned as indicated on the structural formula.

- 10 -

Fig. 4 shows digestion of crosslinked HA hydrogels with hyaluronidase. In Fig. 4A, HA-hydrogels were formed by crosslinking 12mg/ml highly modified (~65-70%) HA-amine (adipic dihydrazido-HA) with 15 mg/ml (SPA)₂-PEG. Gels were incubated with different concentrations of bovine testicular hyaluronidase (HYALase) for the indicated time and the degradation of the gels was measured by the release of glucuronic acid into the supernatant using the carbazole method. (Bitter and Muir, Anal. Biochem. 4, 330-334 (1962)). In Fig. 4B, HA-hydrogels were formed by crosslinking 12 mg/ml optimally modified (~20-25%) HA-amine (adipic dihydrazido-HA) with 15 mg/ml (SPA)₂-PEG (◇); 12 mg/ml highly modified (~65-70%) adipic dihydrazido-HA with 15 mg/ml (SPA)₂-PEG (Δ); 12 mg/ml optimally modified (~20-25%) lysine methylester-HA with either 15 mg/ml (SPA)₂-PEG (Λ) or 0.44 mg/ml glutaraldehyde (□), and 12 mg/ml optimally modified (~10-15%) diaminobutyl-HA with 15 mg/ml (SPA)₂-PEG (o). Gels were incubated with different concentrations of bovine testicular hyaluronidase for the indicated time and the degradation of the gels was measured as in Fig. 4A above.

Fig. 5 shows phase contrast images of cells cultured on different crosslinked HA hydrogels. Fig. 5A: Dedifferentiated chondrocytes cultured on a hydrogel formed from highly modified (~65-70%) HA-amine (adipic dihydrazido-HA) crosslinked with 5 mg/ml (SPA)₂-PEG aggregate as a consequence of inability to adhere to substratum. Fig. 5B: Cells cultured on a hydrogel made up by the same HA-amine crosslinked with 0.25 mg/ml glutaraldehyde show a rounded morphology and no aggregation indicating that they are able to adhere to the substratum. Fig. 5C: Cells cultured on a hydrogel formed from the HA-amine (adipic dihydrazido-HA) modified to a degree of ~20-25% and crosslinked with 2 mg/ml (SPA)₂-PEG adhere to the substratum, spread and subsequently infiltrate the hydrogel. All images show cells 24h post seeding but morphology remains the same even after several days in culture.

Fig. 6 shows *in vivo* evaluation of HA hydrogels formed from different HA derivatives using aldehyde-mediated crosslinking. Subcutaneous implantation in rats of HA hydrogels consisting of (Fig. 6A) 12 mg/ml optimally

- 11 -

modified (~20-25%) HA-amine (adipic dihydrazido-HA) crosslinked with 0.25 mg/ml glutaraldehyde, (Fig. 6B) 7 mg/ml of the same HA-amine crosslinked with 7 mg/ml HA-aldehyde (periodate oxidized), (Fig. 6C) 7 mg/ml of the same HA-amine crosslinked with 7 mg/ml HA-aldehyde (deprotected amino-dimethyl acetal-HA, ~10-15% modified), or (Fig. 6D) 7 mg/ml of the same HA-amine crosslinked with 7 mg/ml HA-aldehyde (deprotected hydrazido-dimethyl acetal-HA, 40-45% modified). The hydrogels also contained 1 mg/ml prefibrillized intact collagen type I, 200 µg/ml BMP-2 and 500 ng/ml IGF-1 to induce bone formation. Tissue specimens were harvested 10 days post implantation, fixed in formalin and processed for histology by paraffin embedding. Sections were stained with Haematoxylin/Eosin. m, matrix material (note: matrix material shrinks during dehydration); s, skin (indicates orientation of implant).

Fig. 7 shows *in vivo* evaluation of HA hydrogels crosslinked with different NHS-esters. Subcutaneous implantation in rats of HA hydrogels consisting of (Fig. 7A) 12 mg/ml highly modified (~65-70%) HA-amine (adipic dihydrazido-HA) crosslinked with 15 mg/ml (SPA)₂-PEG, (Fig. 7B) 12 mg/ml optimally modified (~20-25%) HA-amine (adipic dihydrazido-HA) crosslinked with 15 mg/ml SPA₂-PEG, or (Fig. 7C) 12 mg/ml of the same optimally modified HA-amine crosslinked with 3 mg/ml DTSSP (crosslinker concentrations are equal on a molar basis). The hydrogels also contained 1 mg/ml prefibrillized intact collagen type I, 200 µg/ml BMP-2 and 50 ng/ml TGF-β2 to induce bone formation. Tissue specimens were harvested 10 days post implantation, fixed in formalin and processed for histology by paraffin embedding. Sections were stained with Haematoxylin/Eosin. m, matrix material (note: matrix material shrinks during dehydration); s, skin (indicates orientation of implant).

Fig. 8 shows differential effect of growth factors incorporated into HA hydrogels on tissue transformation. Subcutaneous implantation in rats of the HA hydrogel formed from 12 mg/ml optimally modified (~20-25%) HA-amine (adipic dihydrazido-HA) crosslinked with 15 mg/ml (SPA)₂-PEG. The hydrogels also contained 1 mg/ml prefibrillized intact collagen type I, and were supplemented

- 12 -

either with 200 µg/ml BMP-2 and 500 ng/ml IGF-1 (Fig. 8A), or 200 µg/ml BMP-2 and 50 ng/ml TGF-β2 (Fig. 8B). Tissue specimens were harvested 10 days post implantation, fixed in formalin and processed for histology by paraffin embedding. Sections were stained with Haematoxylin/ Eosin.

5 BRIEF DESCRIPTION OF THE REACTION SCHEMES

Scheme 1 illustrates periodate oxidation of hyaluronic acid.

Scheme 2 illustrates coupling of amines to hyaluronic acid with EDC via an active triazole ester intermediate.

10 Scheme 3 illustrates coupling of amines to hyaluronic acid with EDC via an active *N*-hydroxysuccinimide ester intermediate.

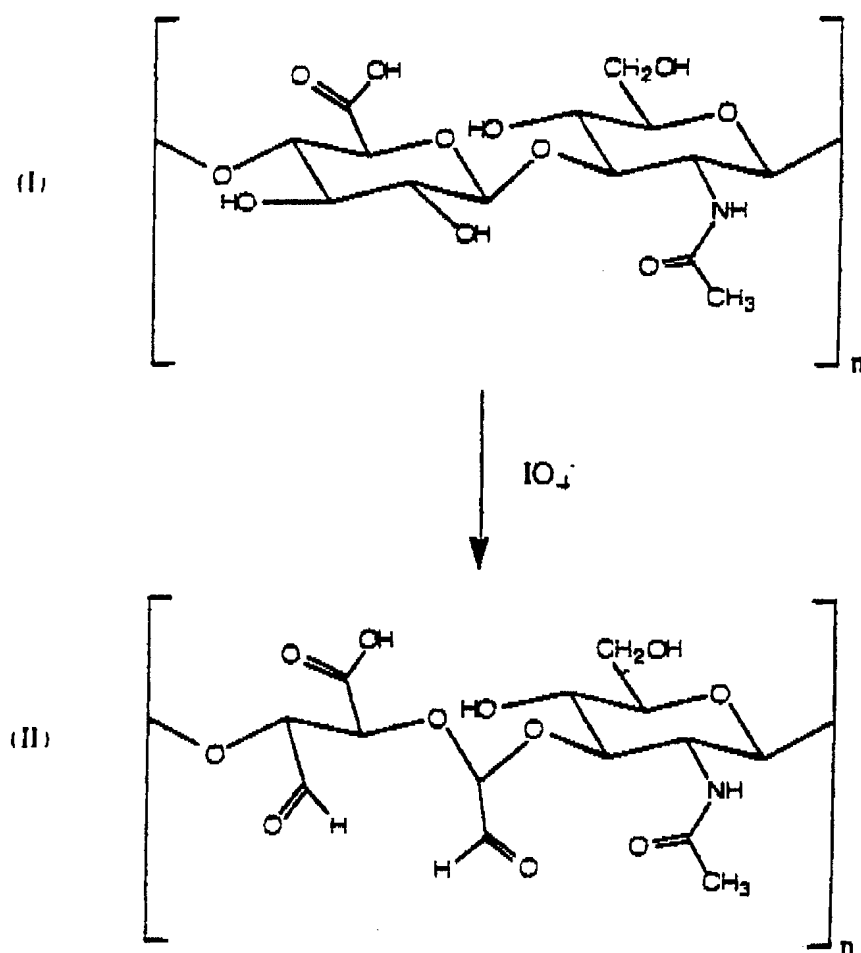
Scheme 4 illustrates crosslinking of amine functionalized hyaluronic acid with various bifunctional *N*-hydroxysuccinimide ester crosslinkers to form hydrogels. (1. (SPA)₂-PEG; 2. DTSSP).

15 Scheme 5 illustrates crosslinking of amine functionalized hyaluronic acid with glutaraldehyde to form hydrogels. In addition to the conventional reaction of aldehydes with amines that results in the formation of a Schiff base, glutaraldehyde is also known to undergo polymerization by aldol condensation yielding polymers with α, β-unsaturated aldehydes at neutral or slightly alkaline pH (Richards and Knowles, J. Mol. Biol. 37, 231-233 (1968)). Subsequent,
20 nucleophilic addition of amines at the ethylenyl double bond creates a stable crosslink.

Scheme 6 illustrates formation of hydrogels with aldehyde functionalized hyaluronic acid. (1. amine functionalized HA; 2. bifunctional amine).

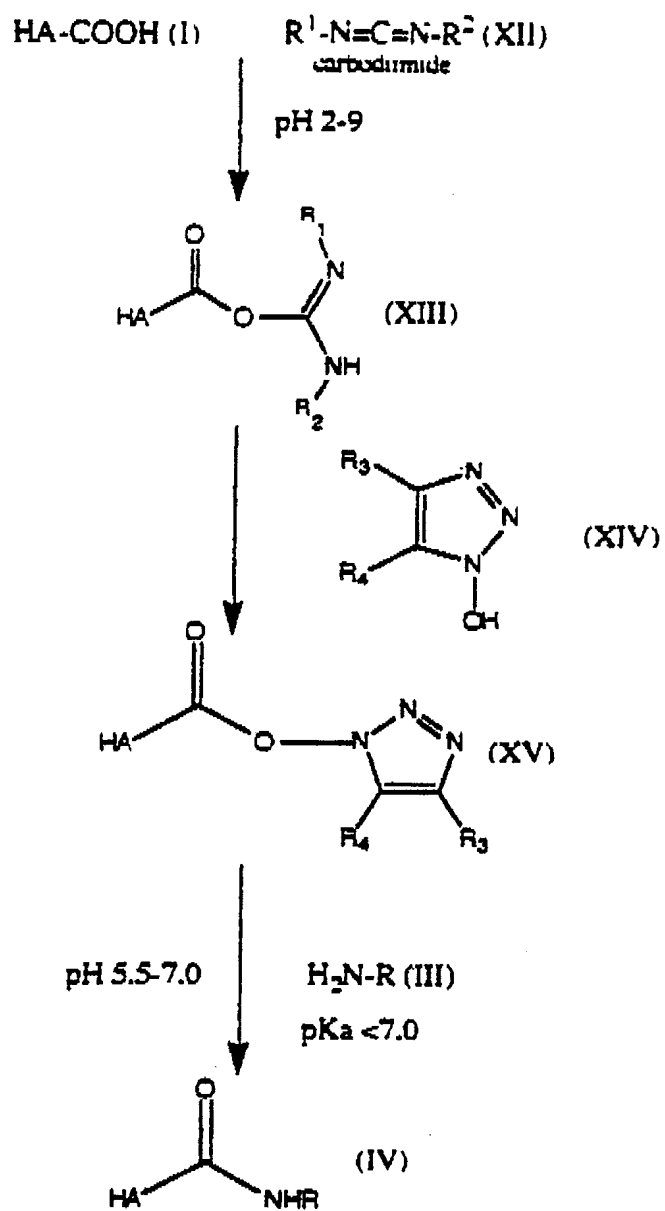
- 13 -

Scheme 1



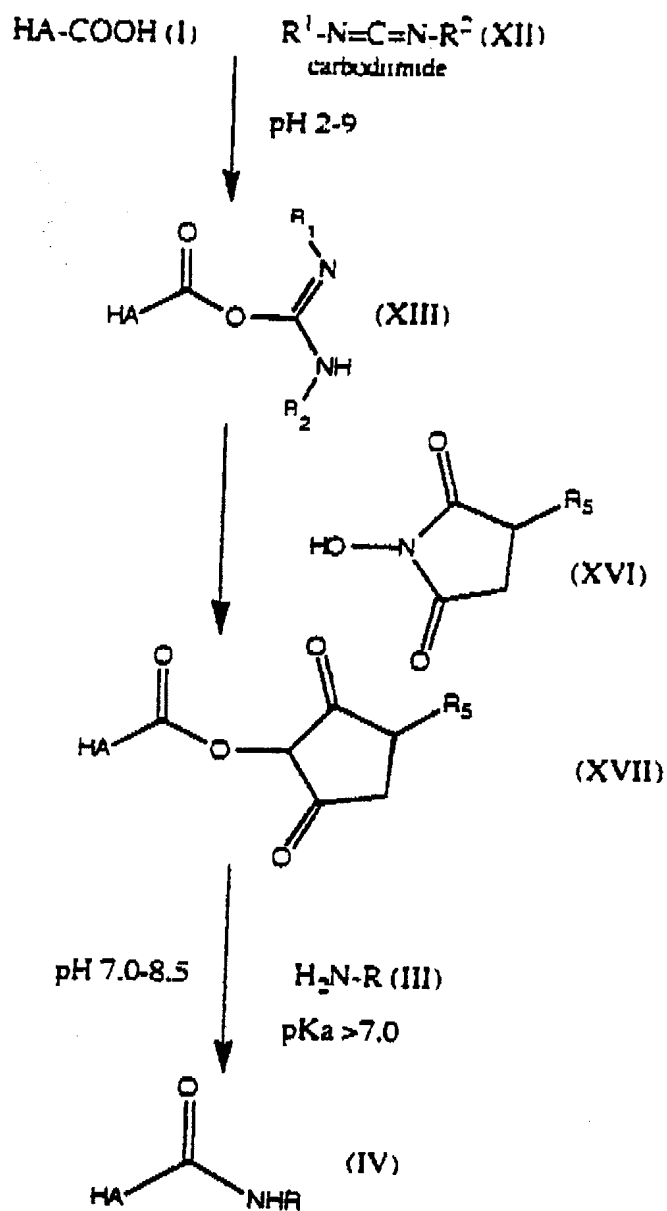
- 14 -

Scheme 2



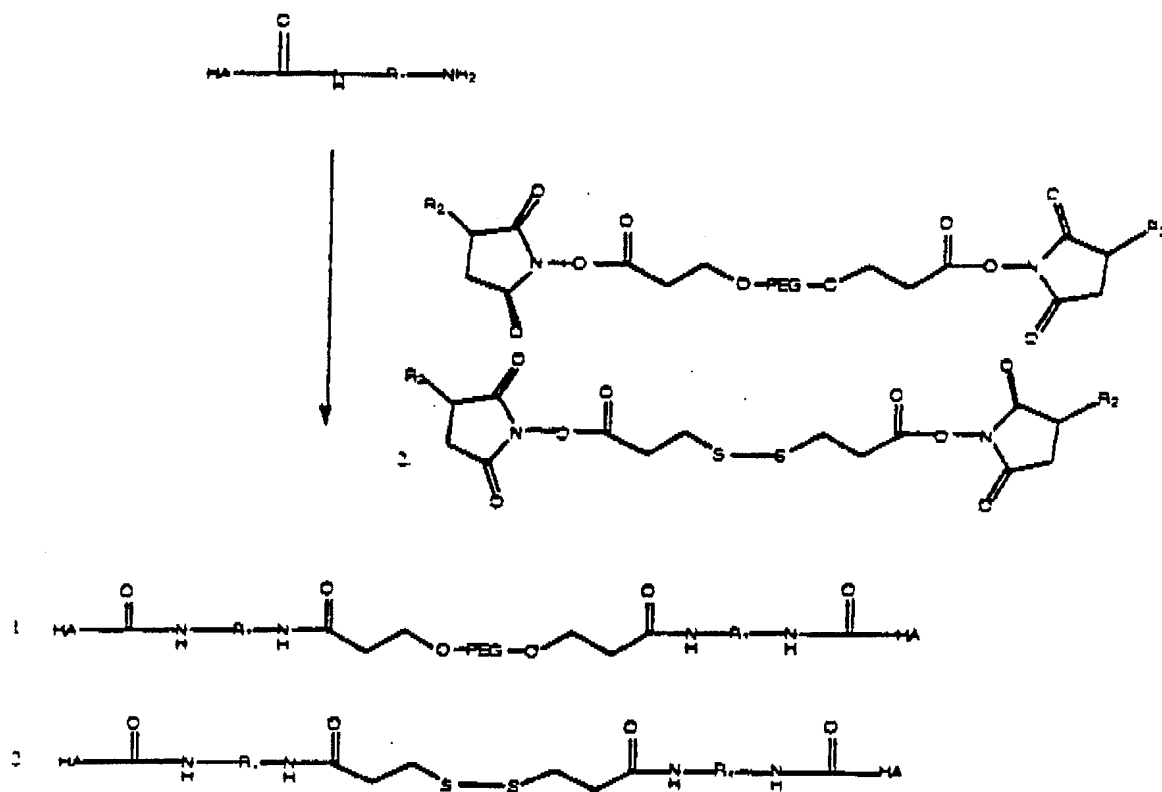
Scheme 3

- 15 -



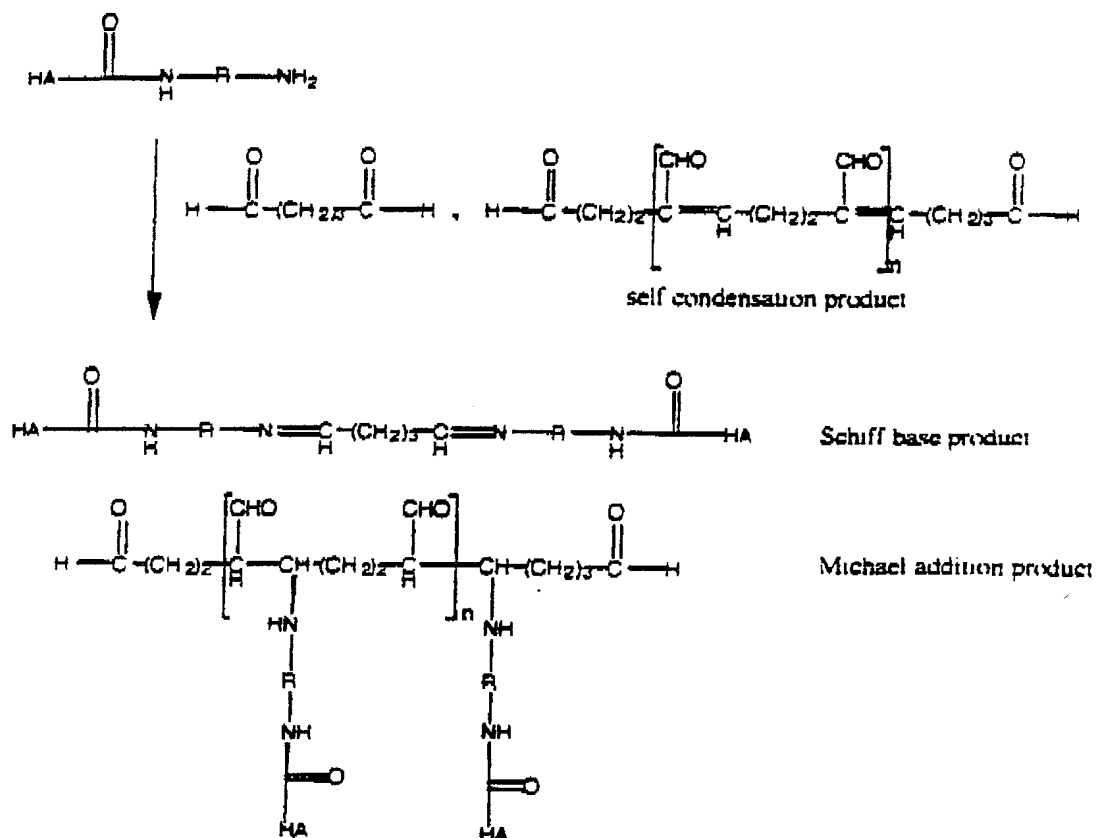
- 16 -

Scheme 4



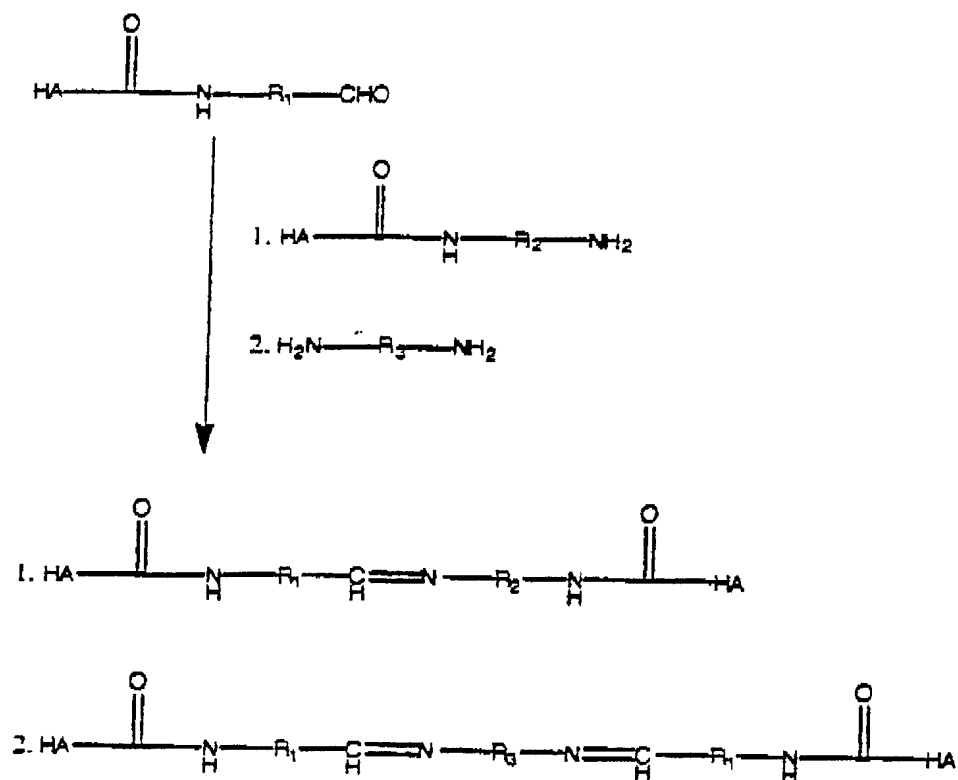
- 17 -

Scheme 5



- 18 -

Scheme 6



- 19 -

DETAILED DESCRIPTION OF THE INVENTION

Using the methods of our invention, we generate an activated form of HA that differs minimally from native HA to conserve its unique physico-chemical properties. We also effect a minimal change affecting only a relatively
5 small number of dissaccharide units of native HA so that we do not alter its property to serve as a cell substratum.

We initially attempted to generate an aldehyde derivative of HA by reduction of the carboxyl groups of the glucuronic acid moieties into aldehydes using 9-borabicyclo-3,3-nonane, a method that allows direct conversion of the
10 carboxylic acid into the aldehyde (Cha et al., Bull Korean Chem. Soc. 9, 48-52 (1988), Cha et al., Org. Prep. Proc. Int. 21, 451-477 (1989)):



However, even though preliminary testing indicated the conversion of the carboxyl groups into aldehydes to a degree of approximately 5-10% (Fig. 1),
15 mixtures of concentrated, viscous HA-aldehyde solutions (~10 mg/ml) with 'small' polyamines such as putrescine, lysine, polylysine, histidine, or polyhistidine did not generate stable gels in a reasonable time frame to be suitable for *in situ* polymerization. It is important to note that the chemical properties of HA are determined not merely by its functional groups *per se* but also by the accessibility of
20 these functional groups of HA in an aqueous environment, which is related to the overall conformational structure and rheological properties of HA. HA behaves like a hydrogel in an aqueous media even in the absence of crosslinks because it forms a network stabilized by hydrogen bonds and van der Waals forces (Laurent and Fraser, *supra*). To increase the accessibility of functional groups, we introduced a
25 spacer between the functional group and the HA chain.

Introducing a functionalized side chain onto HA

We have subsequently developed methodology for introducing side chains into HA by carbodiimide-mediated coupling of primary or secondary amines

- 20 -

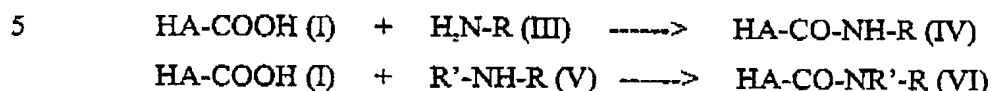
to the carboxyl group of the glucuronic acid moiety using an active ester intermediate. We have used this methodology to generate HA with different terminal functional groups for crosslinking including acetals, aldehydes, amines, and hydrazides. A wide range of functionalized amines are commercially available
5 which allows us to introduce a wide variety of different functional groups useful for crosslinking under physiological conditions using this methodology, including maleimides that react specifically with sulfhydryls or arylazides for photocrosslinking besides the amines and aldehydes described below.

Direct carbodiimide-mediated coupling of amines to the carboxyl
10 group of HA in an aqueous environment, e.g., with EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide), does not yield the predicted product since the *O*-acyl isourea that is formed as a reactive intermediate rearranges rapidly to a stable *N*-acyl urea (Kuo et al., supra). We demonstrate that by "rescuing" the active *O*-acyl isourea by formation of a more hydrolysis resistant and non-rearrangeable
15 active ester intermediate, the coupling of primary amines to HA is possible. A wide variety of active carboxylic esters exist and could be formed for further reaction including NHS-esters, nitrophenol esters, triazole esters, sulfonic esters, etc., as long as the reagent for their preparation is soluble in H₂O or in other polar solvents such as dimethylsulfoxide or dimethylformamide. HA is soluble in H₂O or other
20 aprotic polar solvents in native form and when prepared as a sodium salt or when prepared as a tetrabutylammonium salt as described in U.S. Patent No. 4,957,744, respectively. We have formed active esters of HA with 1-hydroxybenzotriazole (HOBT) or *N*-hydroxysulfo-succinimide using the H₂O soluble carbodiimide EDC for coupling. Nucleophilic addition to the ester formed from HOBT requires the
25 amine to be presented in unprotonated form at acidic pH (about 5.5 to 7.0). Only a limited number of amines including hydrazines and activated amines, e.g., ethylene diamine, have pK_a values in a suitable range and are consequently unprotonated and reactive with the ester-intermediate formed with HOBT (Scheme 2). Simple primary amines, e.g., putrescine, which typically have pK_a values > 9 do not form
30 significant amounts of adduct under acidic coupling conditions. The *N*-

- 21 -

for the coupling reaction to be carried out at neutral pH (about 7.0 to 8.5) and consequently yields products by reaction with simple primary amines (Scheme 3).

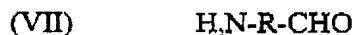
Consequently, this methodology allows for the following reactions to be carried out:



wherein R and R' are alkyl, aryl, alkylaryl or arylalkyl side chains which may contain hetero atoms such as oxygen, nitrogen, and sulfur. The side chain may be branched or unbranched, and be saturated or may contain one or more multiple
 10 bonds. The carbon atoms of the side chain may be continuous or may be separated by one or more functional groups such as an oxygen atom, a keto group, an amino group, and oxycarbonyl group, etc. The side chain may be substituted with aryl moieties or halogen atoms, or may in whole or in part be formed by ring structures such as cyclopentyl, cyclohexyl, cycloheptyl, etc. The side chain may have a
 15 terminal functional group for crosslinking such as aldehyde, amine, arylazide, hydrazide, maleimide, sulfhydryl, etc. The side chain may be a bioactive peptide, e.g., containing receptor binding sites or proteolytic cleavage sites.

Terminal functional groups of the side chain useful for crosslinking of HA under physiological conditions may be selected from the following list:

20 1. aldehydes, see examples



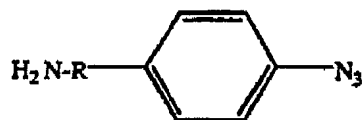
2. amines, see Examples



3. arylazides, e.g., 4-(p-azidosalicylamido) butylamine

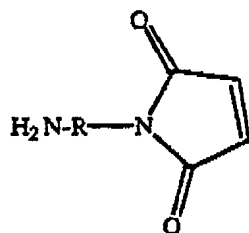
- 22 -

(IX)



4. maleimides, e.g., 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylhydrazide

5 (X)



5. sulfhydryls, e.g., *S*-methylsulfide cysteine

(XI)

 $H_2N-R-SH$

6. peptides, e.g., H_2N -APQQEA, comprising substrate sites for enzymatic crosslinking, e.g., by transglutaminases (Parameswaran et al., Proc. Natl. Acad. Sci. U.S.A. 87, 8472-8475 (1990); Hohenadl et al., J. Biol. Chem. 270, 23415-23420 (1995))

The carbodiimides useful in this reaction may be represented by the following formula:

15 (XII) $R-N=C=N-R'$

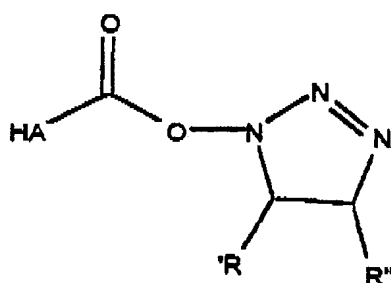
wherein R and R' comprise side chains of variable structure as described above in detail. Carbodiimides which are soluble in an aqueous media are preferred.

- 23 -

The active ester may be of the following class and be formed by carbodiimide-mediated coupling of a compound for preparation of these active esters known to a person in the art:

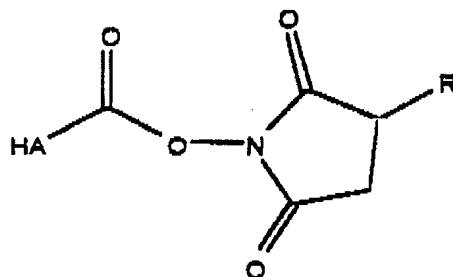
1. triazole esters, *e.g.* 1-hydroxybenzotriazole

5 (XIII)



2. NHS-esters, *e.g.* *N*-hydroxysulfosuccinimide

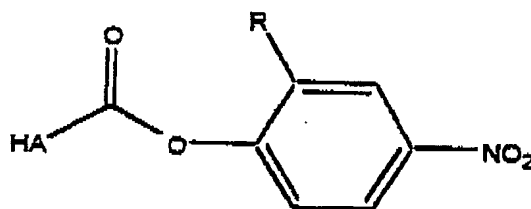
(XIV)



- 24 -

3. nitrophenol esters, *e.g.* *p*-nitrophenol

(XV)

Preparation of HA-aldehyde derivatives

5 A side chain containing a protected aldehyde in the form of an acetal was prepared as follows. *N*-(2,2-dimethoxyethyl)-4-(methoxycarbonyl) butanamide was obtained from aminoacet-aldehyde dimethyl acetal and mono-methyl succinate using EDC coupling. An amino group for the coupling to HA

10 was subsequently introduced by reacting the product with hydrazine, yielding the desired side chain with the protected aldehyde, *N*-(2,2-dimethoxyethyl)-4-(hydrazido)butanamide. The side chain was coupled to HA using HOBT and EDC (Scheme 2). An acetal side chain with a simple primary amine, 1-aminoethyl-dimethylacetal, was conjugated to HA using *N*-hydroxysuccinimide and EDC (Scheme 3). The HA-derivatives were purified by ethanol precipitation. The

15 nature of the HA-derivatives was confirmed by ¹H NMR (Fig. 2). The HA-acetal derivatives are easily activated to the reactive aldehydes by mild acid treatment. Other HA-aldehyde derivatives with variations in the length of the side chain have been prepared in a similar manner. See Examples 1-3.

Preparation of HA-amine derivatives

20 Diaminoethane, lysine methyl ester, histidine, and adipic, succinic or suberic dihydrazide was coupled to HA using HOBT and EDC (up to 5 fold excess depending on the desired degree of modification) and adjusting the pH to

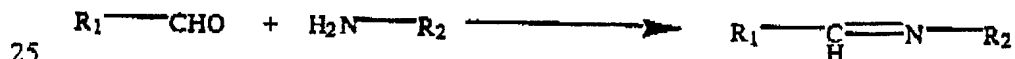
- 25 -

- ~6.5 by repeated addition of 0.1M HCl during the reaction (Scheme 2). HA-derivatives were also prepared in a similar manner using *N*-hydroxy-sulfosuccinimide and primary amines containing unconjugated amino groups with a higher pKa (>9) such as 1,4-diaminobutane or 1,6 diaminohexane (Scheme 3).
- 5 The HA derivatives were purified by repeated ethanol precipitation or by extensive dialysis, and the nature of the HA derivatives was confirmed by ¹H NMR (Fig. 3). The degree of modification was calculated from the NMR data and found to be as high as 70%. Reaction conditions were subsequently adjusted such that a degree of modification of approximately 20% was achieved. Limiting the amount of
- 10 carbodiimide proved to be most successful in controlling the degree of modification. A degree of modification of 10-25% yielded efficient crosslinking but also a molecule that would still be recognized by glycosidases and by HA receptors as HA and thus allow for recognition and processing of the material by cells (see below). Similar HA derivatives were synthesized using succinic, adipic
- 15 or suberic dihydrazide or diaminoethane, -butane, or -hexane to study the effect of the length of the spacer separating the introduced functional group from the HA-chain on the subsequent crosslinking. See Examples 4-8.

Crosslinked HA hydrogels

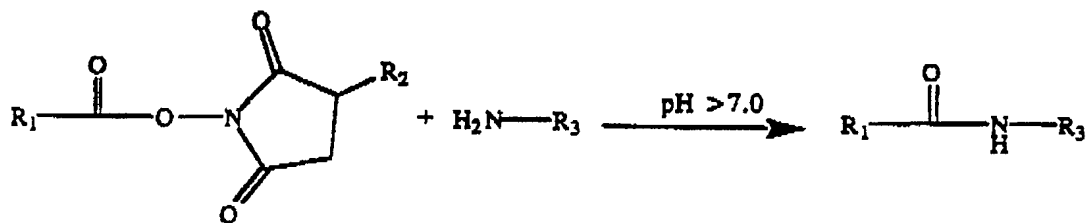
- The functionalized HA molecules can be crosslinked by reacting
- 20 HA derivatives with different functionalities or using homo- or heterobifunctional crosslinkers which are available in large variety. The following basic reaction schemes are suitable for crosslinking of the described forms of modified HA (see Examples 9-12):

1. aldehyde-mediated crosslinking

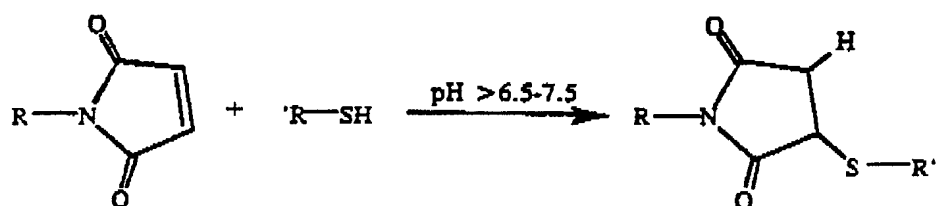


2. active ester-mediated crosslinking

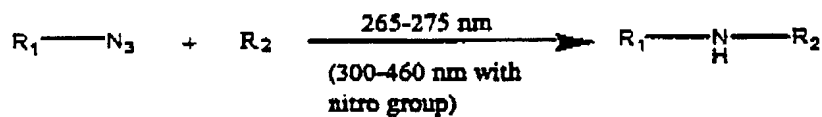
- 26 -



3. maleimide-mediated crosslinking

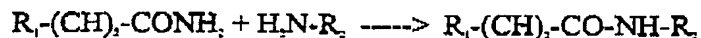


4. photo-crosslinking



5

5. enzymatic crosslinking (transglutaminase)



- Crosslinking of the HA-amine derivatives ($M_r \sim 10^6$) with bifunctional active esters, e.g., polyethyleneglycol-bis-succinimidyl-propionate [(SPA)₂-PEG] and reducible 3,3'-dithiobis(sulfo-succinimidyl-propionate) (DTSSP) (Scheme 4), or bifunctional aldehydes, e.g. glutaraldehyde (Scheme 5), generated excellent hydrogels. Stable gels could be formed by crosslinking 5 to 25

- 27 -

derivative with >0.05 mM aldehyde or >0.2 mM active ester (numbers are reflecting functional group concentrations). Optimal gels were generated by crosslinking 10-15mg/ml HA derivative, modified to a degree of about 10-25%, with about 0.2mM aldehyde or 0.6mM active ester. Similarly, crosslinking of the HA-aldehyde derivatives ($M_r \sim 10^6$) (optimally about 10-15mg/ml) with bifunctional amines (optimally about 0.2mM) yielded excellent gels (Scheme 6). Conjugated amines such as dihydrazines or benzylamines are required for *in situ* polymerization of HA in this case to resonance stabilize the unstable Schiff base product formed by reaction of an aldehyde with a primary amine (i.e., hydrazines yield a more stable hydrazone linkage). Hydrogels were also formed from an equimolar mixture of HA-aldehyde derivatives and the different HA-amine derivatives (Scheme 6). Optimal gels were formed with ~ 15 mg/ml of the HA derivatives. At the optimal concentrations of HA and crosslinker, gelation occurred typically in about 30 sec. to 5 min. which is suitable for *in situ* polymerization. The crosslinked HA hydrogels were sensitive to glycosidases, i.e., testicular hyaluronidase, indicating that they are biodegradable (Fig. 4).

A number of different tests including cell culture assays and animal experiments served to assess biocompatibility of the formulated biomaterials. Embedding of chondrocytes into the polymerizing HA hydrogels showed that neither aldehyde nor NHS-ester-mediated crosslinking was toxic to cells at the concentrations employed. Seeding of cells on top of prepolymerized HA hydrogels showed a wide variety of cellular behaviours depending on the nature of the crosslinker and crosslinking density (Fig. 5). Highly crosslinked HA hydrogels were impenetrable to cells (Fig. 5, A and B), while optimally crosslinked gels were infiltrated (Fig. 5C). Supplementation of the HA hydrogels with cell adhesion molecules such as fibronectin (in the range of 0.1 to 1 mg/ml) did induce adhesion and spreading of cells on the materials independent of the nature of the crosslinker and the crosslinking density, but did not change the results with regard to cell infiltration, demonstrating that the lack of infiltration is due to the high crosslinking density and not the absence of cell-matrix interactions. See below and Fig. 7.

- 28 -

Subcutaneous implantation of biomaterials in rats is the established model for evaluation of biocompatibility of biomaterials (Laurencin et al., J. Biomed. Mat. Res. 24, 1463-1481 (1990)) and for induction of ectopic bone formation by members of the TGF- β gene family, and bone morphogenetic proteins (BMP) in particular (Wang et al., Proc. Natl. Acad. Sci. U.S.A. 87,2220-2224 (1990); Sampath *et al.*, J. Biol. Chem. 267, 20352-20362 (1992)). Taking into consideration the cell culture results, we have formulated a number of HA hydrogels for *in vivo* biocompatibility testing in this model. Implantation of prepolymerized HA hydrogel discs loaded with recombinant BMP-2 and IGF-1 or TGF- β 2 subcutaneously in rats showed a mild fibrosis with a varying degree of cartilage and bone formation depending on the nature of the HA biomaterial (Figs. 6 and 7). The growth factors were mixed with the HA derivatives prior to gelling and the induction of bone formation suggests that neither reaction mechanism used for HA crosslinking (aldehyde or active ester-mediated reactions) significantly affected the biological activity of the growth factors. Little inflammation was observed with active ester crosslinked HA-amine derivatives (Fig. 7) or with HA-amine derivatives crosslinked with various HA-aldehyde derivatives (Fig. 6B-6D) while a stimulation of foreign body giant cells was seen when the same HA-amine derivatives were crosslinked with glutaraldehyde (Fig. 6A). The degree of modification of RA strongly affected the resorption and transformation rate of the biomaterials (Fig. 7A, 7B). Nevertheless, limited bone formation was seen even with a biomaterial formed from a highly modified (65-70%) HA-amine derivative (Fig. 7A). The absence of bone formation with a smaller bifunctional NHS-ester crosslinker indicates that the size of the generated crossbridge is crucial for resorption and cellular infiltration (Fig. 7C). This is probably due to the difference in pore size of the material formed with crosslinkers of different sizes. The infiltration and transformation rate was similar with BMP-2/IGF-1 and BMP-2/TGF- β 2 loaded biomaterials, indicating that the resorption rate is a material property. However, at ten days post-implantation, the newly formed tissue was largely cartilage in the first group and largely bone in the second group (Fig. 8), exemplifying the angiogenic

- 29 -

effect of TGF- β 2 (Yang and Moses, J. Cell. Biol., 111, 731-741 (1990)). This demonstrates that the biological activity of the HA material can be modulated by inclusion of different bioactive factors. The lack of significant adverse effects and the demonstration of the desired biological activity of these novel HA biomaterials
5 *in vivo* demonstrates their usefulness as a delivery vehicle for cells and growth factors in the field of tissue regeneration.

There are several approaches to the production of HA, including extraction from tissue and biosynthesis. Extraction from tissue typically uses fresh or frozen cocks' combs (U.S. Pat. No. 5,336,767), although other tissues including
10 the synovial fluid of joints (Kvam et al., Anal. Biochem. 211, 44-49 (1993)), human umbilical cord tissue, bovine vitreous humor, and bovine tracheae, have been used. It is also possible to prepare HA by microbiological methods, such as by cultivating a microorganism belonging to the genus *Streptococcus* which is anhemolytic and capable of producing HA in a culture medium (U.S. Pat. Nos. 4,897,349;
15 4,801,539; 4,780,414; 4,517,295; 5,316,926). The HA raw material for preparing the compositions of the invention preferably consists of high molecular weight HA, more preferably of molecular weight greater than 0.5 million daltons, and more preferably of molecular weight greater than one million daltons. The HA raw material for the compositions of examples of this invention described herein was
20 obtained from Genzyme Corp. (Cambridge, MA), and had a molecular weight greater than one million daltons. The size of the HA was unchanged after derivatization.

The compositions of the invention have many therapeutic uses. The fact that the compositions may be cured in a surgically practical time frame of one
25 to five minutes *in situ* with concurrent crosslinking to the tissue surfaces allows for employment as a tissue glue. Many situations in various surgical applications require such adhesives. For example, the compositions of the invention may be used to stem hemorrhage in general surgery, reconstruct nerves and vessels in reconstructive, neuro- and plastic surgery, and to anchor skin, vascular, or cartilage
30 transplants or grafts in orthopedic, vascular, and plastic surgery. Those of skill in

- 30 -

the art may choose and design particular embodiments of the invention which are particularly suitable for a desired application, by adjusting several factors, including: (1) the degree of functionalization of HA, which affects the crosslinking density of the material and interaction with cellular proteins, including receptors and glycosidases; (2) the concentration of the crosslinker, which affects the crosslinking density of the material; (3) the size of the generated cross-bridge, which affects the pore size of the material; (4) the nature of the crosslinking mechanism, which determines polymerization time and the specificity of the reaction; and (5) the nature of the cross-bridge, which provides biological cues. See Figs. 4, 5, and 7 for data concerning HA hydrogels with different crosslinking densities and pore sizes. Generally, active ester- or photo-crosslinking are preferred to form materials for applications requiring fast gelation and strong bonding with tissue surfaces, such as tissue glues. Materials with anti-adhesive properties, which are useful to form tissue separations or for tissue augmentation, are formed from highly modified HA derivatives with low molecular weight crosslinkers, which generates a dense material with very small pores, thereby minimizing cell adhesion and infiltration. Conversely, biodegradable scaffolds for tissue repair are formed from HA with a limited degree of derivativization and high molecular weight crosslinkers, which generate a porous, biodegradable material. The crossbridge may even contain biological cues, such as peptide sequences, which facilitate material degradation by, for example, proteolysis or cellular infiltration (e.g., the RGD sequence).

Compositions of this invention were designed to serve as a vehicle for the delivery of cells or bioactive molecules such as growth factors to stimulate focal repair. The crosslinked HA derivatives are porous hydrogels in which biologically or therapeutically active compounds (e.g., growth factors, cytokines, drugs, and the like) can be physically or chemically incorporated. These compounds will then be subject to sustained release by chemical, enzymatic, and physical erosion of the hydrogel and/or the covalent linkage between the HA chain and biologically active compound over a period of time. Local delivery of growth factors with such a scaffold facilitates wound healing and tissue regeneration in

- 31 -

many situations. For example, the compositions of the invention may be used not only to promote bone formation and stimulate cartilage repair in orthopedic procedures, as described more fully below, but also to treat pathological wound conditions such as chronic ulcers. They may also serve as a scaffold to generate
5 artificial tissues, e.g., cartilage (Hauselmann et al., Am. J. Physiol. 271, C742-752 (1996)), through proliferation of autologous cells in culture. Similar procedures for generation of equivalents of other tissues or organs, including skin, liver, and others, in culture may be developed in the future and may be used in combination with the compositions of the invention.

10 Highly crosslinked materials have an anti-adhesive property with respect to cells, and such compositions may be used to generate tissue separations and to prevent adhesions following surgery. See Figs. 5A and 7C, showing highly modified HA-amine, i.e., adipic dihydrazido HA, preferably crosslinked with low molecular weight bifunctional NHS-ester. The viscoelastic properties of HA make
15 it particularly well suited for this purpose, and it is used clinically to achieve temporal pain relief by repeated intraarticular injections in arthropathies as a "joint lubricant", and as a protective agent for eye irritations and in ophthalmic surgery. The technique of tissue separation is used in facial reconstruction in plastic surgery and dentistry. Prevention of the formation of adhesions is particularly relevant in
20 reconstructive surgery of tendons, in surgical procedures in the urogenital system, and in thoracic surgery. Many different HA-based materials are already in clinical use in these areas. (See products manufactured by Anika Therapeutics, Inc. (Woburn, MA), Biomatrix, Inc. (Ridgefield, NJ), Genzyme Corp. (Cambridge, MA), and Fidia, S.p.A. (Abano Terme, Italy)). Those of skill in the art may choose
25 and design particular embodiments of the invention which are particularly suitable for a desired application by selecting distinct features as outlined above.

 The injectable nature of the compositions of the invention also renders them suitable for tissue augmentation in plastic surgery, where the HA matrix serves primarily as a biocompatible filler material, e.g., for filling dermal
30 creases or lip reconstruction. Again, those of skill in the art may choose and design

- 32 -

particular embodiments of the invention which are particularly suitable for a desired application, as outlined above.

The half-life of pharmacological compounds, both synthetic and biological, has been shown to be drastically increased when delivered in a form conjugated to HA (Larsen and Balazs, Adv. Drug Delivery Rev. 7, 279-293 (1991); Drobnik, J., Drug Delivery Rev. 7, 295-308 (1991)). The functionalized forms of HA provided by this invention allow for easy substitution with pharmacologically active agents, such as anti-inflammatories, analgesics, steroids, cardiovascular agents, anti-tumor agents, immunosuppressants, sedatives, anti-bacterial, anti-fungal, and anti-viral agents, etc., and may be used for sustained drug release over time, either locally in hydrogel form or systemically in free form.

In orthopedic surgery, the functionalized forms of HA of this invention have applications as a tissue glue or bioactive matrix material in the treatment of chondral and osteochondral fractures, osteochondritis dissecans, meniscal tears, as well as ruptured ligaments, tendons, or myotendinous junctions. The HA materials of this invention may serve to facilitate anchorage of chondral or osteochondral transplants or grafts, or other biological or artificial implant materials, or to stimulate new bone or cartilage formation by serving as a scaffold for cells or as a delivery vehicle for growth factors. One general approach to promote articular cartilage repair based on the compositions of the invention comprises using: (1) *in situ* polymerized HA hydrogel as a matrix to fill the defect which is to be repaired and to provide a scaffold for repair cells, (2) an optional chemotactic agent to attract repair cells to the matrix and defect site, or alternatively, autologous chondrocytes or mesenchymal stem cells, (3) an optional factor to promote cellular proliferation of repair cells in the matrix and defect site; (4) sustained release of a transforming factor by the HA hydrogel over time to promote differentiation of the repair.

Throughout this specification and claims, the word "comprise," or variations such as "comprises" or "comprising," will be understood to imply the inclusion of a

- 33 -

stated integer or group of integers but not the exclusion of any other integer or group of integers.

In order that this invention be more fully understood, the following examples are set forth. These examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention in any way.

Example 1

Preparation of N-(2,2-Dimethoxyethyl)-4-

(methoxycarbonyl)butanamide (1) - EDC (4.98g, 0.026mol) was added to a mixture of aminoacetaldehyde dimethyl acetal (2.18ml, 20mmol) and methyl monoester of succinic acid (2.64g, 20mmol) in 75ml of dichloromethane, and the reaction mixture stirred for 24 h at room temperature. The solution was extracted successively with 50ml each of ice cold solutions of 0.75M sulfuric acid, 1M NaCl, saturated sodium bicarbonate, and 1M NaCl. The organic phase was collected and dried with sodium sulfate. The solvent was evaporated under reduced pressure yielding a syrup, which showed a single spot on charring upon TLC in solvent A (R_f 0.75) and solvent B (R_f 0.24). The apparent yield of 1 was 65%. ¹H NMR in CDCl₃, δ 5.70 (bs, 1H, NH), 4.34 [t, 1H, CH-(OCH₃)], 3.67 (s, 3H, COOCH₃), 3.43-3.35 (s and t, 8H, CH₂OC and CHCH₂NH), 2.38-2.26 (m, 4H, CH₂CO).

Formation of Acyl-hydrazide (2) from 1 - Anhydrous hydrazine

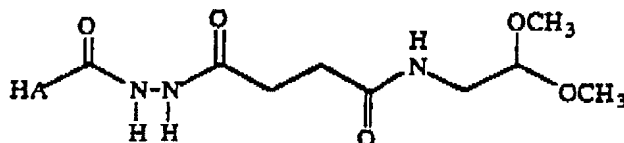
(248μl, 7.9mmol) was added to a solution of 1 (1.73g, 7.9mmol) in 5ml of anhydrous methanol. The mixture was stirred at room temperature overnight and the solvent subsequently evaporated under reduced pressure yielding a solid residue. The residue was dissolved in H₂O (6ml) and extracted three times with an equal volume of dichloromethane. The aqueous solution was evaporated to dryness under reduced pressure and then further dried overnight in vacuo. The crystalline solid (1.04g, 82% yield) was homogeneous on TLC in solvent A (R_f 0.10) when visualized by charring. The ¹H NMR spectrum indicated the loss of the ester methoxy group when compared to 1.

- 34 -

Preparation of Hydrazido-dimethyl acetal-HA (formula XIX) -

Sodium hyaluronate (100mg, 0.25mmol) and *N*-(2,2-dimethoxyethyl)-4-(hydrazido)butanamide (2) (1.646g, 7.5mmol) was dissolved in H₂O (40ml, 2.5mg/ml HA). The pH was adjusted to 6.5 and HOBT (169mg, 1.25mmol) predissolved in a 1:1 mixture of water and DMSO (1ml) and EDC (240mg, 1.25mmol) was added and the reaction mixture was stirred overnight. The pH was subsequently adjusted to 7.0 with 1M NaOH and NaCl added to produce a 5% w/v solution. HA was precipitated by addition of three volume equivalents of ethanol. The precipitate was redissolved in H₂O at a concentration of approximately 5mg/ml and the precipitation repeated twice. The purified product was freeze dried and kept at 4°C under N₂. See Fig. 2B for NMR data of the product.

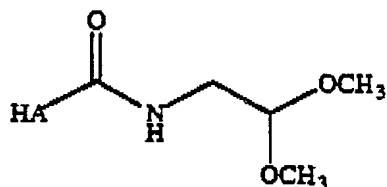
(XIX)

**Example 2**

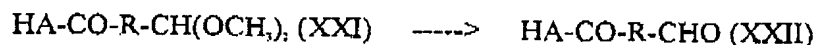
Preparation of Aminoacetaldehyde-dimethyl acetal-HA (formula XX) - Sodium hyaluronate (100mg, 0.25mmol) and 2,2-dimethoxyethylamine (0.788g, 7.5mmol) was dissolved in H₂O (40ml, 2.5mg/ml HA). The pH was adjusted to 7.5 and NHS.SO₃Na (268mg, 1.25mmol) and EDC (240mg, 1.25mmol) was added and the reaction mixture was stirred overnight. The pH was subsequently adjusted to 7.0 with 1M NaOH and NaCl added to produce a 5% w/v solution. HA was precipitated by addition of three volume equivalents of ethanol. The precipitate was redissolved in H₂O at a concentration of approximately 5mg/ml and the precipitation repeated twice. The purified product was freeze dried and kept at 4°C under N₂.

- 35 -

(XX)

**Example 3**

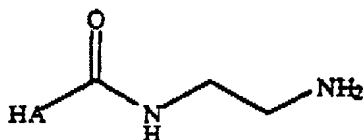
Deprotection of HA-acetals to form HA-aldehydes - The acetal modified HA (formula XXI) was dissolved in H₂O to a concentration of 5-10mg/ml and 1M HCl was added to give a final concentration of 0.025M. The solution was then allowed to stand at room temperature for 0.5 to 1.0h. The solution was neutralized by the addition of 1M NaOH, yielding the deprotected HA-aldehyde (formula XXII).

**Example 4**

Preparation of Diaminoethane-HA (formula XXIII) - Sodium hyaluronate (100mg, 0.25mmol) and 1,2-diaminoethane HCl (0.998g, 7.5mmol) was dissolved in H₂O (40ml, 2.5mg/ml HA). The pH was adjusted to 6.5 and HOBT (169mg, 1.25mmol) predissolved in a 1:1 mixture of water and DMSO (1ml) and EDC (240mg, 1.25mmol) was added and the reaction mixture was stirred overnight. The pH was subsequently adjusted to 7.0 with 1M NaOH and NaCl added to produce a 5% w/v solution. HA was precipitated by addition of three volume equivalents of ethanol. The precipitate was redissolved in H₂O at a concentration of approximately 5mg/ml and the precipitation repeated twice. The purified product was freeze dried and kept at 4°C under N₂.

- 36 -

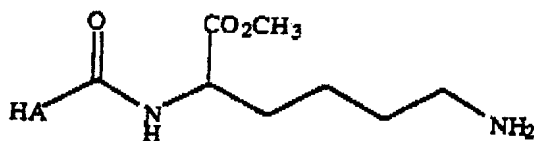
(XXIII)

**Example 5**

Preparation of L-Lysine methyl ester-HA (formula XXIV) - Sodium

- 5 hyaluronate (100mg, 0.25mmol) and L-lysine methyl ester dihydrochloride (1.748g, 7.5mmol) was dissolved in H₂O (40ml, 2.5mg/ml HA). The pH was adjusted to 6.5 and HOBT (169mg, 1.25mmol) predissolved in a 1:1 mixture of water and DMSO (1ml) and EDC (240mg, 1.25mmol) was added and the reaction mixture was stirred overnight. The pH was subsequently adjusted to 7.0 with 1M NaOH and NaCl
- 10 added to produce a 5% w/v solution. HA was precipitated by addition of three volume equivalents of ethanol. The precipitate was redissolved in H₂O at a concentration of approximately 5mg/ml and the precipitation repeated twice. The purified product was freeze dried and kept at 4°C under N₂. See Fig. 3C for NMR data of the product.

15 (XXIV)

**Example 6**

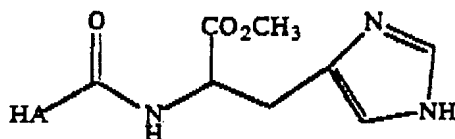
Preparation of L-Histidine methyl ester HA (formula XXV) -

- Sodium hyaluronate (100mg, 0.25mmol) and L-histidine methyl ester
- 20 dihydrochloride (1.815g, 7.5mmol) was dissolved in H₂O (40ml, 2.5mg/ml HA). The pH was adjusted to 6.5 and HOBT(169mg, 1.25mmol) predissolved in a 1:1

- 37 -

mixture of H₂O and DMSO (1ml) and EDC (240mg, 1.25mmol) was added and the reaction mixture was stirred overnight. The pH was subsequently adjusted to 7.0 with 1M NaOH and NaCl added to produce a 5% w/v solution. HA was precipitated by addition of three volume equivalents of ethanol. The precipitate was redissolved in H₂O at a concentration of approximately 5mg/ml and the precipitation repeated twice. The purified product was freeze dried and kept at 4°C under N₂. See Fig. 3B for NMR data of the product.

(XXV)



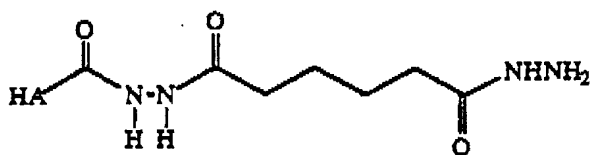
10 Example 7

Preparation of Hydrazido-HA (formula XXVI) - Sodium

hyaluronate (100mg, 0.25mmol) and dihydrazide *i.e.* adipic dihydrazide (1.31g, 7.5mmol) was dissolved in H₂O (40ml, 2.5mg/ml HA). The pH was adjusted to 6.5 and HOBT (169mg, 1.25mmol) predissolved in a 1:1 mixture of water and DMSO (1ml) and EDC (240mg, 1.25mmol) was added and the reaction mixture was stirred overnight. The pH was subsequently adjusted to 7.0 with 1M NaOH and NaCl added to produce a 5% w/v solution. HA was precipitated by addition of three volume equivalents of ethanol. The precipitate was redissolved in H₂O at a concentration of approximately 5mg/ml and the precipitation repeated twice. The purified product was freeze dried and kept at 4°C under N₂. See Fig. 3D for NMR data of the product.

- 38 -

(XXVI)

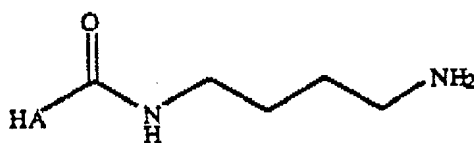
**Example 8***Preparation of Diaminoalkyl-HA (formula XXVII) - Sodium*

5 hyaluronate (100mg, 0.25mmol) and a diaminoalkane, *i.e.* 1,2-diaminobutane HCl (1.208g, 7.5mmol) was dissolved in H₂O (40ml, 2.5mg/ml HA). The pH was adjusted to 7.5 and NHS.SO₃Na (268mg, 1.25mmol) and EDC (240mg, 1.25mmol) was added and the reaction mixture was stirred overnight. The pH was subsequently adjusted to 7.0 with 1M NaOH and NaCl added to produce a 5% w/v

10 solution. HA was precipitated by addition of three volume equivalents of ethanol. The precipitate was redissolved in H₂O at a concentration of approximately 5mg/ml and the precipitation repeated twice. The purified product was freeze dried and kept at 4°C under N₂. See Fig. 3A for NMR data of the product.

(XXVII)

15

**Example 9***Formation of crosslinked HA hydrogels - The general procedure*

for forming crosslinked HA hydrogels is as follows: Modified HA is dissolved by agitation in H₂O or phosphate buffered saline (pH 7.4-8.5) at a concentration of 5-

20 25mg/ml. The degree of modification of the HA derivative is derived from the integration of the ¹H NMR peaks. After complete dissolution, the HA derivative solution is transferred to a 1 ml syringe. When reacting the HA derivatives with low molecular weight crosslinkers, a slight excess of the compound (about 1.1

- 39 -

molar equivalent of functional groups) is dissolved in a second 1 ml syringe in 1/10 of the HA derivative volume immediately prior to use. The syringes are connected while paying special attention to excluded air, the contents are rapidly mixed, typically with 20 passages, and then extruded. When reacting HA derivative

5 molecules with different functionalities, 0.5-1.0 equivalent of HA-aldehyde is mixed with equivalent of HA-hydrazine, depending on the degree of modification of HA derivatives. At room temperature, gelation occurs within about 30 seconds to several minutes, depending on the formulation, and the gel properties do not significantly change after approximately 5 minutes.

10 Example 10

Digestion of crosslinked HA hydrogels with hyaluronidase - The general procedure for digestion of crosslinked HA hydrogels is as follows: HA hydrogels are formed in 1ml syringes by crosslinking 12mg/ml HA-amine in phosphate buffered saline with various crosslinkers as indicated in Fig. 4. Gelling is

15 allowed to occur for 1 hour at 37°C for the reaction to be complete, after which identical ~100µl cylindrical gels are formed by cutting the syringes with a razor blade. The gels are incubated with different concentrations of bovine testicular hyaluronidase (Sigma) 50-5000U/mL in 400µl of 30mM citric acid, 150mM Na₂HPO₄, pH 6.3, 150mM NaCl for the indicated time 0-48 hours. Degradation of

20 the gels was determined from the release of glucuronic acid into the supernatant as measured using the carbazole method (Bitter and Muir, Supra).

Example 11

Crosslinked HA hydrogels as a matrix for cell culture -

25 Chondrocytes were isolated from bovine nose cartilage according to established procedures (Häuselmann et al., Matrix 12, 116-129 (1992; Küttner et al., J. Cell Biol. 93, 743-750 (1982)), cultured in Ham's F12 medium containing 5% fetal bovine serum and antibiotics, and dedifferentiated by monolayer culture on plastic. For cytotoxicity studies, cells (2.5x10⁵) were embedded into the HA hydrogels by

- 40 -

gently mixing the trypsinized cells (about 50 to 100 μ l) with the polymerizing HA and crosslinker mixture (approximately 400 μ l gel volume) prior to complete setting. Agarose embedded cells served as a control. After adaptation to the culture conditions (24h), cell proliferation and metabolic activity was assessed by pulse
5 labeling with [3 H]thymidine and [35 S]methionine. For cell infiltration studies, HA hydrogels were polymerized in 24-well plates (~15mm diameter and 3mm height) for 1h at room temperature, and extensively rinsed with phosphate buffered saline. Cell adhesion molecules or chemotactic factors, *e.g.* IGF-1, were added to the HA solution prior to crosslinking when desired. After 24h, cells (2.5×10^5) were seeded
10 on top of the HA-hydrogels and cultured as above. At different time points post seeding, gels were fixed in phosphate buffered 4% paraformaldehyde and processed for paraffin embedding. Cell infiltration was assessed by staining sections with Haematoxylin/Eosin. See Fig. 5.

Example 12

15 *Subcutaneous implantation of HA hydrogels in rats* - Rats (2-3 per test material) were anaesthetized with ketamine/xylazine, the ventral thorax and abdomen shaved, and prepared aseptically. A small vertical incision was made on either side of the xiphoid cartilage of the sternum and the skin undermined with a blunt instrument to separate the skin from the underlying tissue. HA hydrogels
20 were polymerized in 3ml syringes as described. For induction of chondro-osseous differentiation, 1mg/ml prefibrillized intact collagen type I (Organogenesis, Canton, MA), 200 μ l/ml recombinant BMP-2 (Genetics Institute, Cambridge, MA), and 500 ng/ml IGF-1 (Celtrix Pharmaceuticals, Santa Clara, CA) or 50ng/ml TGF- β 2 (Celtrix Pharmaceuticals, Santa Clara, CA) were mixed with the HA solution prior
25 to crosslinking. Collagen fibrils were prepared by slow polymerization (from dilute solutions of 2-3mg/ml) of acid-solubilized collagen in phosphate buffered saline and harvested by centrifugation following standard protocols (McPherson et al., Collagen Rel. Res. 5, 119-135 (1985)). Gelling of the HA hydrogels was allowed to occur for 24h at room temperature for the reaction to be complete, after which

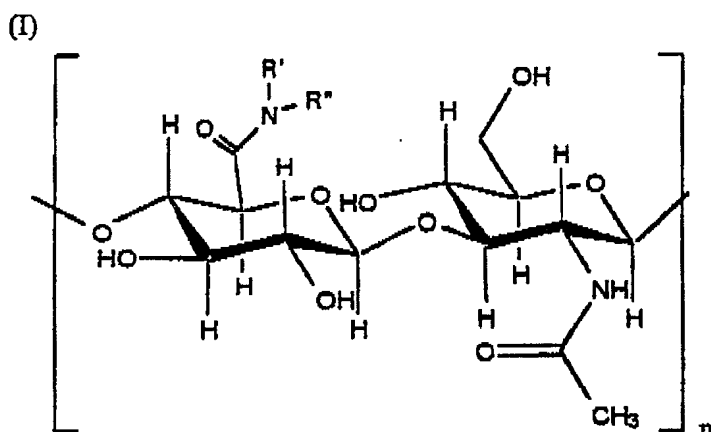
- 41 -

identical ~3mm thick cylindrical gels were prepared by cutting the syringes with a razor blade. HA hydrogel discs were then placed in each pocket and the skin incisions closed with sutures. Ten days post operatively, the rats were euthanized and the appearance of the implant sites, *i.e.* degree of inflammation, grossly
5 examined and tissue specimens harvested and processed for histology by fixation in phosphate buffered formalin and paraffin embedding. Sections were stained with Haematoxylin/Eosin and with Safranin-O/fast green, and cell infiltration and transformation (cartilage and bone formation) induced by the biomaterial as well as signs of fibrosis and inflammation in the surrounding tissue evaluated. See Figs.
10 6-8.

- 42 -

We claim:

1. A composition comprising derivative of hyaluronic acid comprising disaccharide subunits, wherein at least one of said disaccharide subunits is a substituted disaccharide subunit having a substitution at a carboxyl group, such that the substituted disaccharide subunit is of the formula

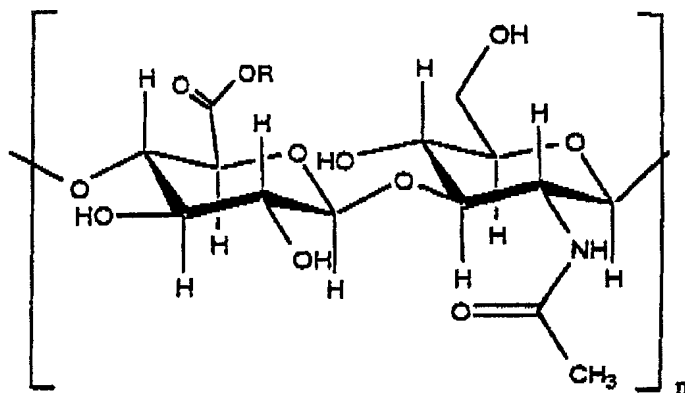


- wherein R' and R'' is a side chain comprising one or more functional groups selected from the group consisting of hydrogen; bioactive peptide; alkyl; aryl; alkylaryl; arylalkyl substituted alkylaryl containing an atom or atoms of oxygen, nitrogen, sulfur, or phosphorous; substituted arylalkyl containing an atom or atoms of oxygen, nitrogen, sulfur; phosphorous, halogen, or metal ion; and substituted heterocycle containing an atom or atoms of oxygen, nitrogen, sulfur; phosphorous, halogen or metal ion; and
- 15 said side chain functional groups being bound directly to each other or separated by a member selected from the group consisting of ether, keto, amino, oxycarbonyl, sulfate, sulfoxide, carboxamide, alkyne and alkene; and
- said side chain terminating with a terminal functional group selected from the group consisting of hydrogen, peptide, aldehyde, amine, arylazide, hydrazide, maleimide, sulfhydryl, active ester, ester, carboxylate, imidoester,
- 20 halogen and hydroxyl.

- 43 -

2. The composition of claim 1, wherein at least 5% of said disaccharide subunits are substituted disaccharide subunits.
3. The composition of claim 1, wherein at most 95% of said disaccharide subunits are substituted disaccharide subunits.
- 5 4. The composition of claim 1, wherein at most 5% of said disaccharide subunits are substituted disaccharide subunits.
5. The composition of claim 1, wherein at least one of said terminal functional groups is selected from the group consisting of peptide, aldehyde, amine, arylazide, hydrazide, maleimide, sulfhydryl, and active ester, whereby said
- 10 composition is amenable to crosslinking.
6. The composition of claim 1, wherein the molecular weight of said composition is at least 100,000 daltons.
7. The composition of claim 1, wherein the molecular weight of said composition is at most 100,000 daltons.
- 15 8. The composition of claim 1, wherein the molecular weight of said composition is at least 1,000,000 daltons.
9. The composition of claim 1, wherein said composition is water soluble.
10. A composition comprising an activated ester of hyaluronic acid
- 20 of the formula:

(II)



- 44 -

wherein R is selected from the group consisting of a substituted triazole, N-sulfosuccinimide, nitrophenol, partially halogenated phenol, perhalophenol, and pentafluorophenol.

11. A hydrogel of crosslinked HA derivatives, wherein said HA
5 derivatives are compositions according to claim 1.

Next, a set of claims to the hydrogels.

12. A hydrogel of crosslinked HA derivatives, wherein said HA
derivatives are selected from the group consisting of the compositions of claim 10.

13. The hydrogel of crosslinked HA derivatives of claim 11, wherein
10 said hydrogel is biodegradable.

14. A method for making a derivative of hyaluronic acid, comprising
the steps of:

- a) forming an activated ester at a carboxylate of a
glucuronic acid moiety of hyaluronic acid; and
15 b) substituting at the carbonyl carbon of the activated ester
formed in step (a), a side chain comprising a nucleophilic portion and a functional
group portion.

15. The method of claim 14, wherein the nucleophilic portion is
selected from the group consisting of ammonia, primary amine, secondary amine,
20 hydroxyl, and sulfhydryl.

16. The method of claim 14, wherein the functional group portion is
selected from the group consisting of active ester, aldehyde, amine, arylazide,
hydrazide, maleimide, sulfhydryl, and peptide.

17. The method of claim 14, wherein step (a) is performed with an
25 active ester selected from the group consisting of a substituted triazole,
N-sulfosuccinimide, nitrophenol, partially halogenated phenol, perhalophenol,
pentalouorophenol, HOBT, and NHS, by carbodiimide-mediated coupling.

18. The method of claim 14, comprising the additional step of: (c)
forming a cross-linked hydrogel from the hyaluronic acid derivative.

- 45 -

19. A method for forming a matrix for a temporary scaffold for tissue repair according to the method of claim 18, wherein the crosslinker is selected from the group consisting of polyvalent active ester, aldehyde, amine, arylazide, maleimide, and sulfhydryl.
- 5 20. A method for forming a matrix for a temporary scaffold for tissue repair according to the method of claim 18, wherein the HA derivative comprises a peptide substrate for transglutaminase, and wherein the HA derivative is crosslinked using transglutaminase.
- 10 21. The method of claim 18, wherein step (c) is performed in the presence of cells.
22. The method of claim 18, wherein step (c) is performed in the presence of at least one member selected from the group consisting of growth factors, cytokines, drugs, and bioactive peptides.
23. The method of claim 22, wherein the bioactive peptide is RGD.
- 15 24. The method of claim 22, wherein the bioactive peptide is a substrate for transglutaminase.
25. The method of claim 24, wherein the bioactive peptide is APQQEA.
26. The method of claim 24, wherein the growth factor is TGF- β or BMP.
- 20 27. The method of claim 18, wherein step (c) is performed *in situ* in a patient in need of tissue repair.
28. A tissue adhesive comprising a hydrogel of claim 11, wherein the side chain is selected from the group consisting of activated ester, aldehyde, arylazide, and maleimide.
- 25 29. A tissue adhesive comprising an HA derivative of claim 10.
30. A tissue adhesive comprising a hydrogel of claim 11, wherein the crosslinked HA derivatives are formed using a cross-linker selected from the group consisting of polyvalent active ester, aldehyde, arylazide, and maleimide.

- 46 -

31. A tissue adhesive comprising a hydrogel of claim 11, wherein the cross-linked hydrogel is formed in the presence of at least one member selected from the group consisting of growth factors, cytokines, drugs, and bioactive peptides.
- 5 32. A tissue adhesive of claim 31, wherein the growth factor is TGF- β or BMP-2.
33. A matrix for cell structures comprising a hydrogel of claim 11, wherein the crosslinked HA-derivatives are formed using a cross-linker selected from the group consisting of polyvalent active ester, aldehyde, amine, arylazide, maleimide, and sulfhydryl.
- 10 34. A matrix for cell structures comprising a hydrogel of claim 11, wherein the crosslinked hydrogel is formed in the presence of at least one member selected from the group consisting of growth factors, cytokines, drugs, and bioactive peptides.
- 15 35. A matrix for cell cultures according to claim 34, wherein the growth factor is TGF- β or BMP-2.
36. A matrix for a scaffold comprising a hydrogel of claim 11, wherein the crosslinked HA-derivatives are formed using a cross-linker selected from the group consisting of polyvalent active ester, aldehyde, amine, arylazide, maleimide, and sulfhydryl.
- 20 37. A matrix for a scaffold comprising a hydrogel of claim 11, wherein the crosslinked hydrogel is formed in the presence of at least one member selected from the group consisting of growth factors, cytokines, drugs and bioactive peptides.
- 25 38. A matrix for a scaffold according to claim 37, wherein the growth factor is TGF- β or BMP-2.
39. The matrix of claim 37, wherein the matrix further comprises cells.

Fig. 1

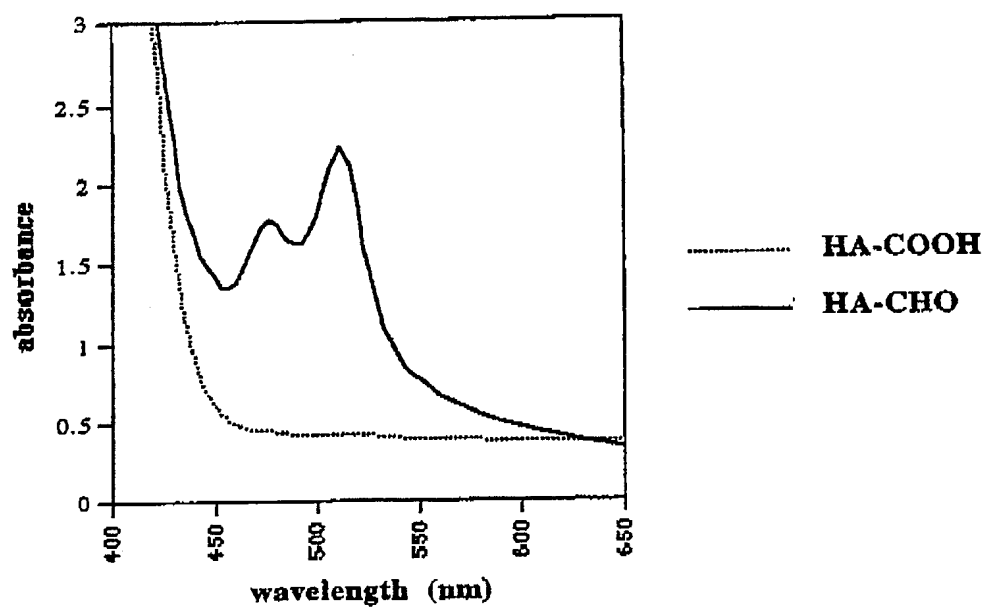


Fig. 2A

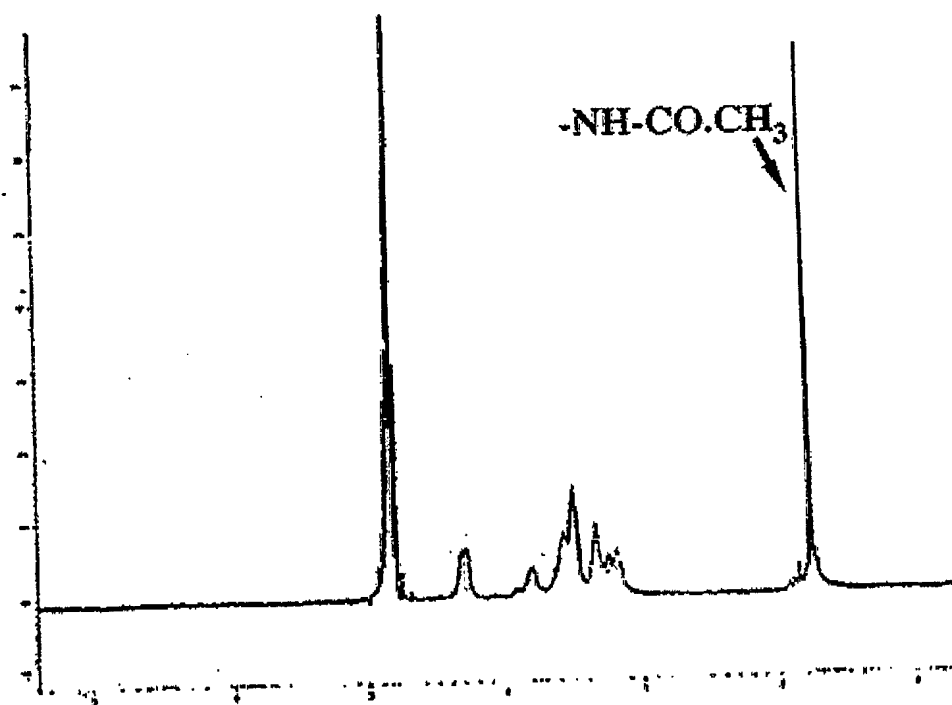
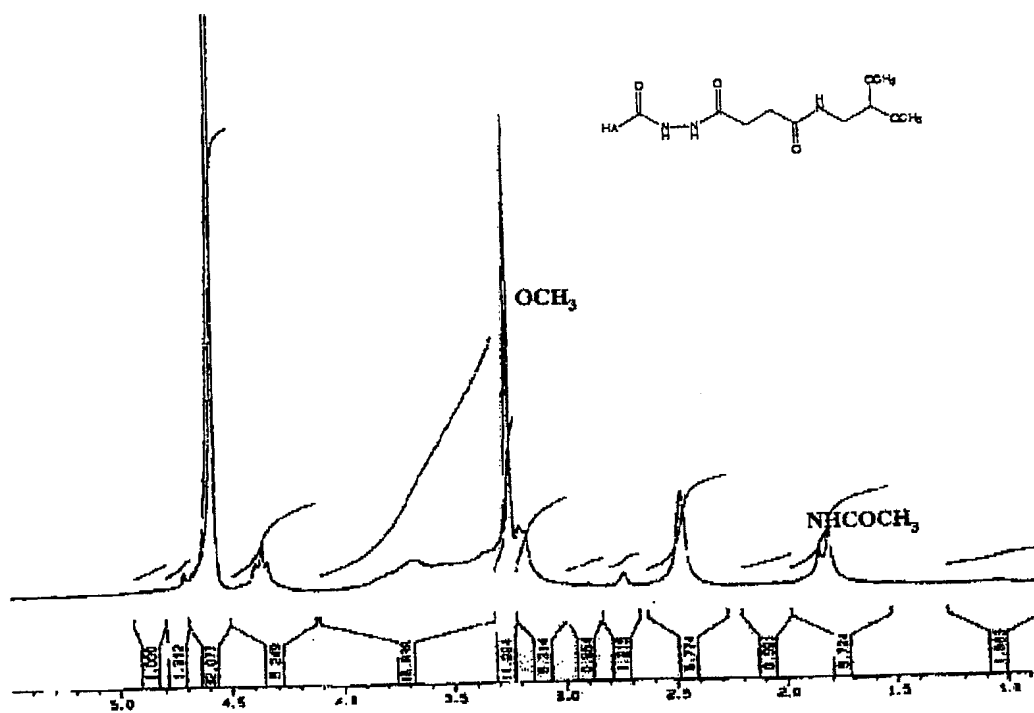
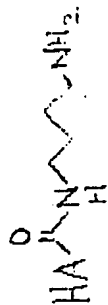


Fig. 2B





A. Panitch PCAB 7 D20 *** H0374707.300

Fig. 3A

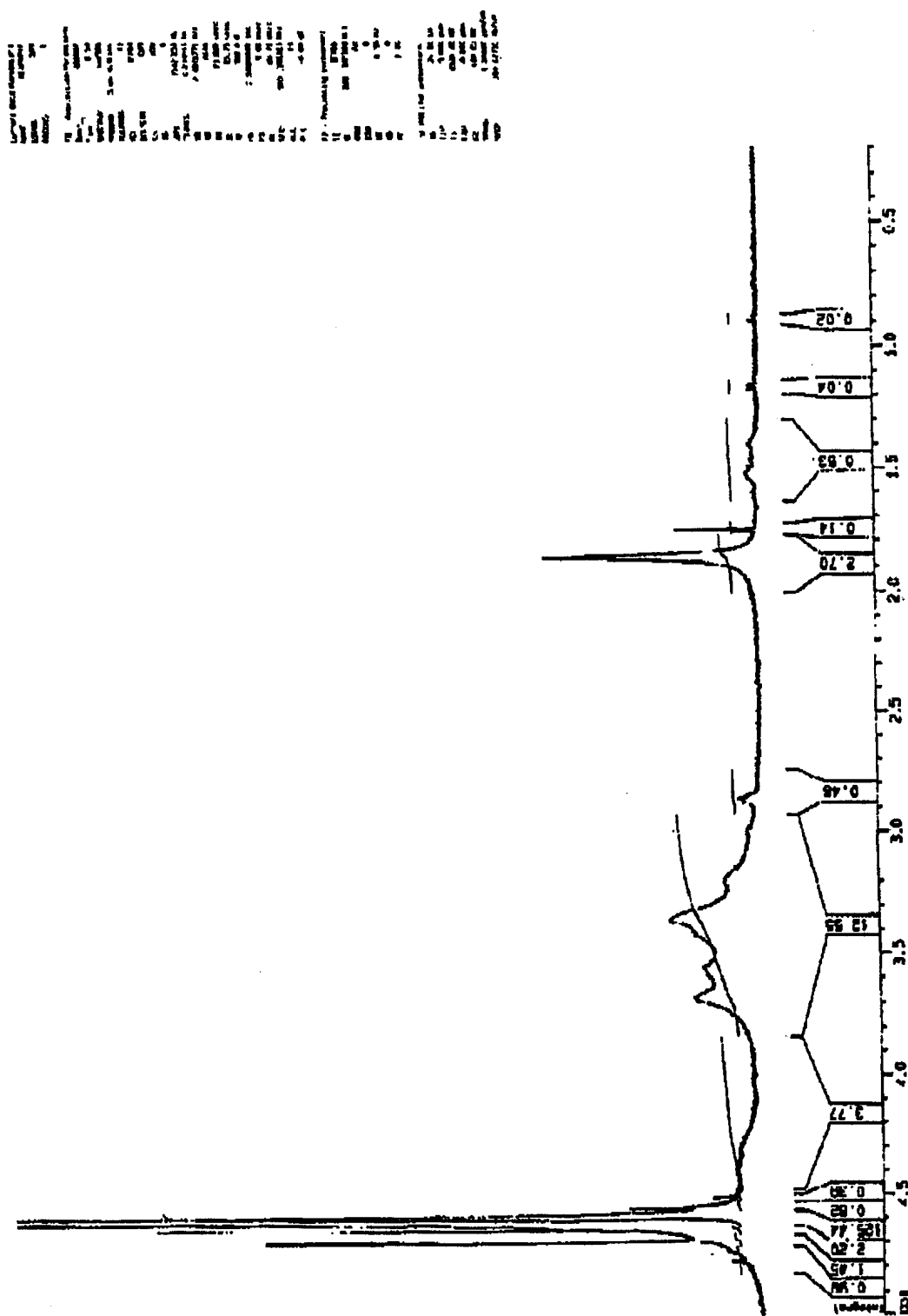
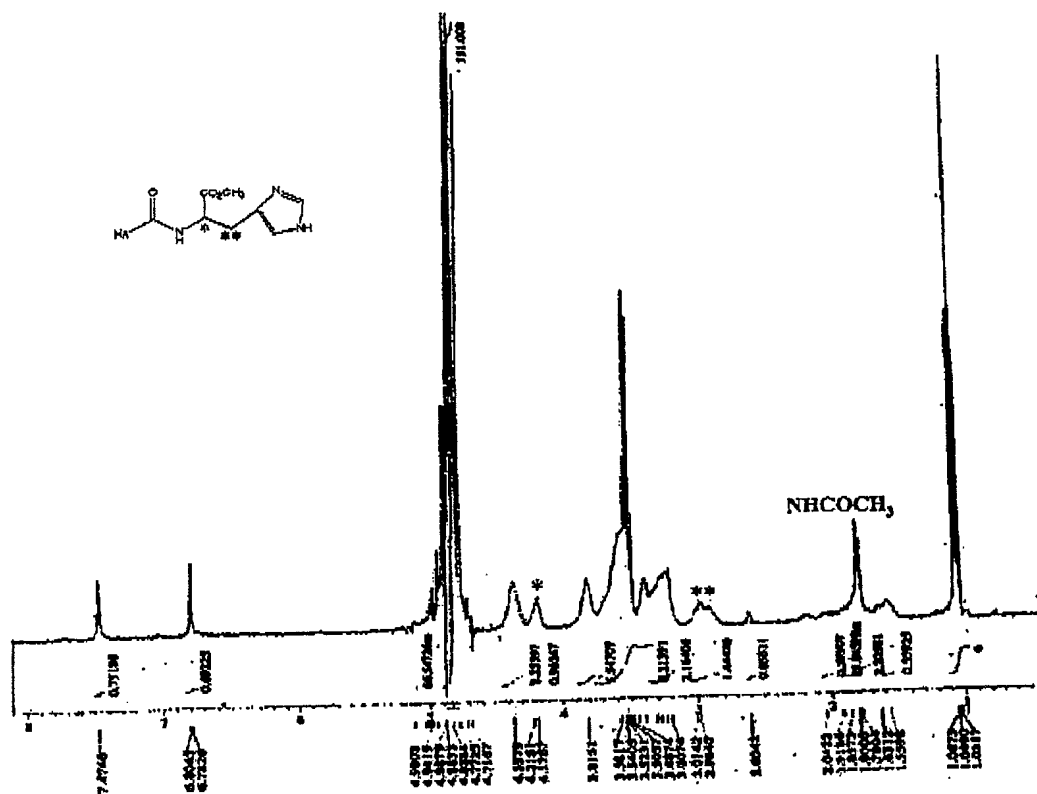


Fig. 3B





Pratt PCAB 2 D20 /// NMR H0354607.300

Fig. 3C

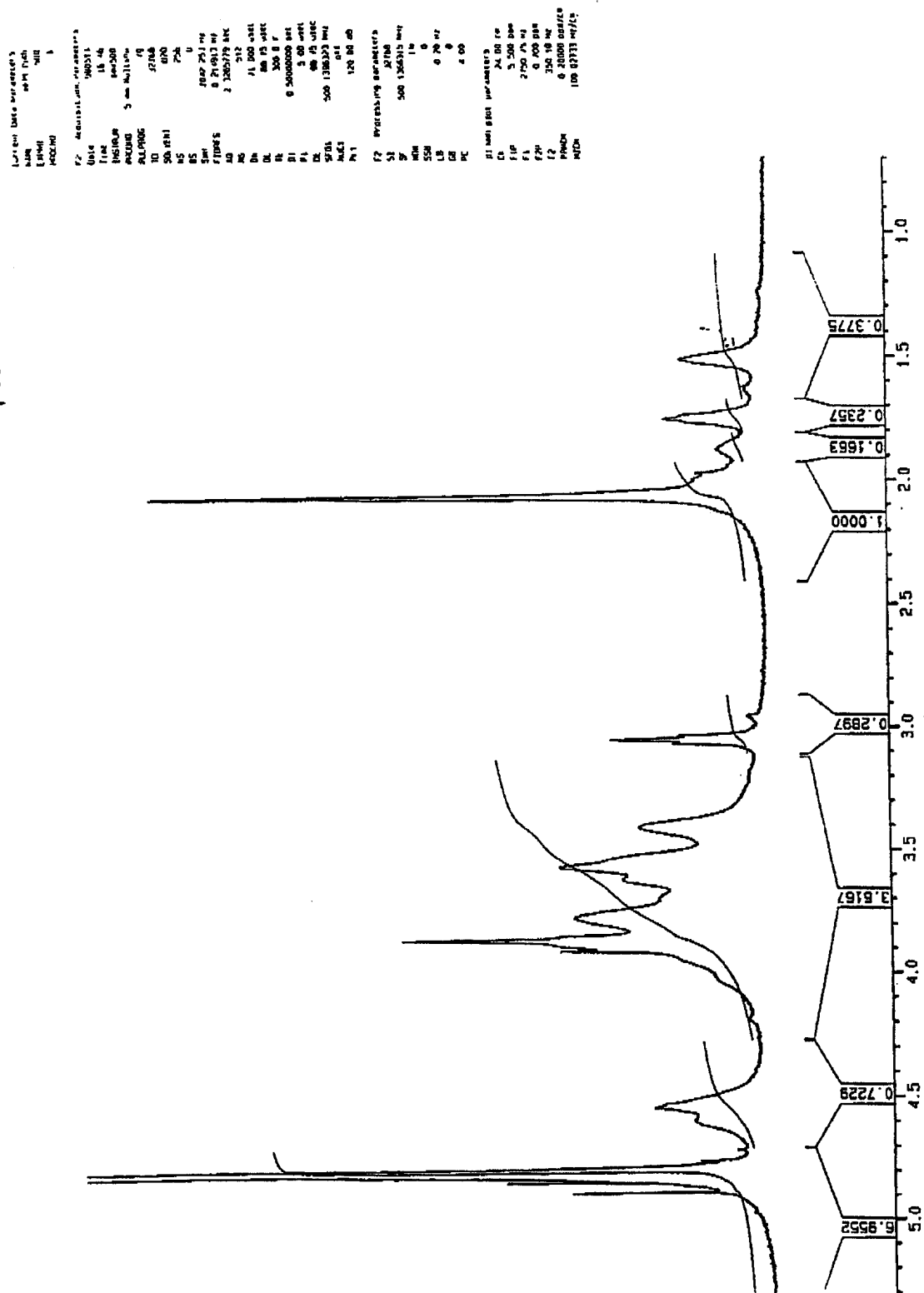


Fig. 3D

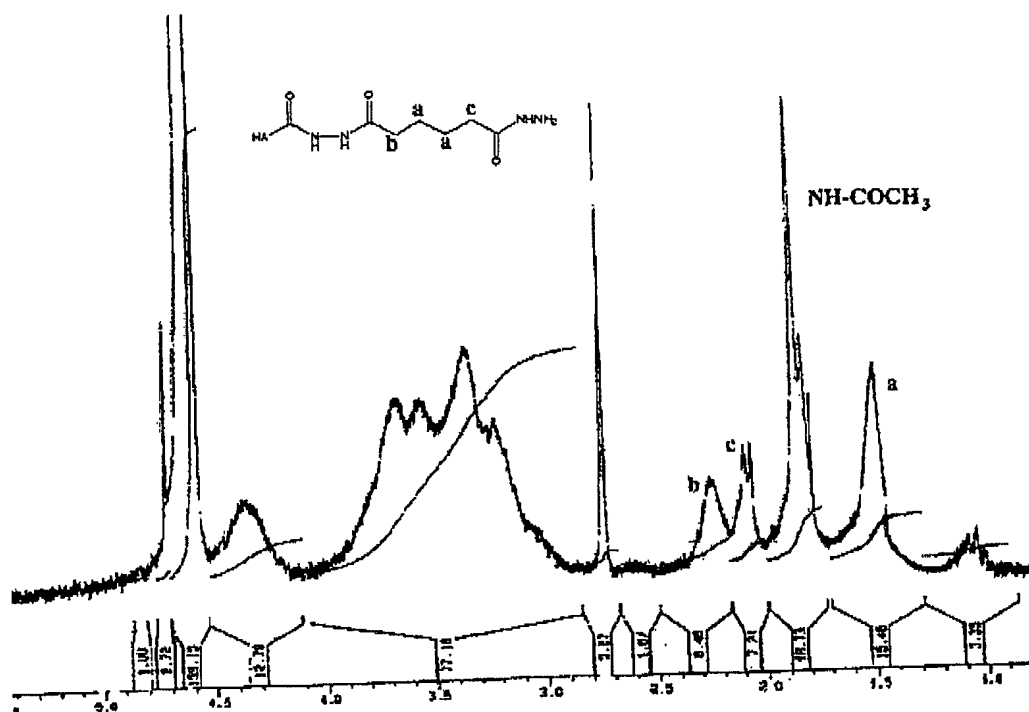


FIG. 4A

A

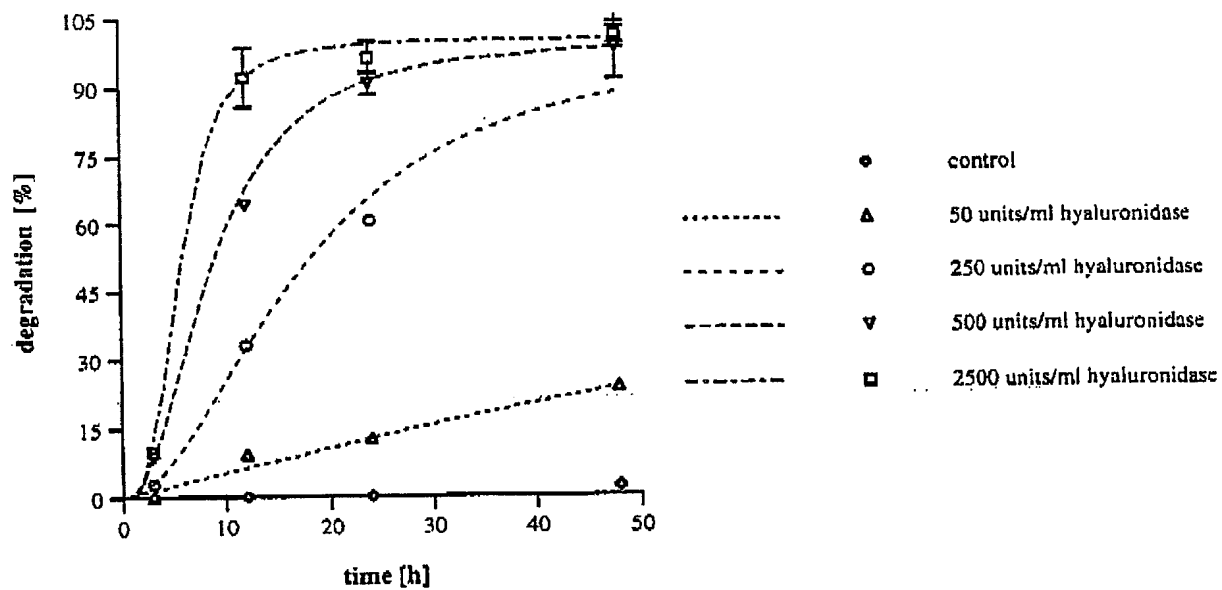


FIG. 4B

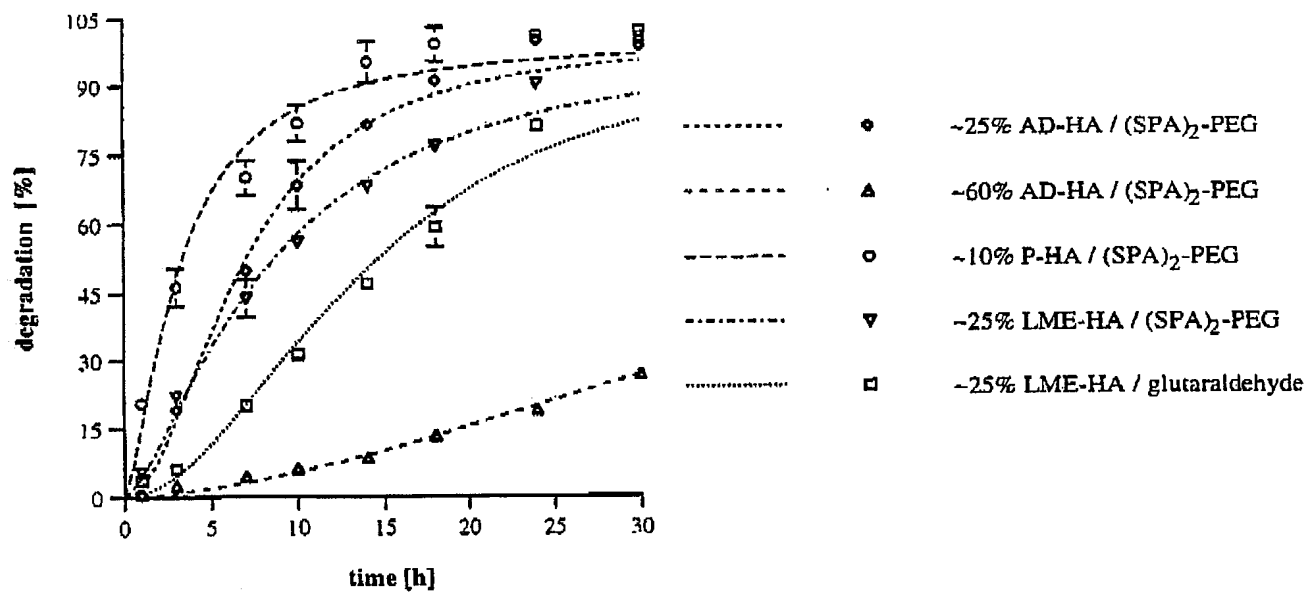
B

Fig. 5

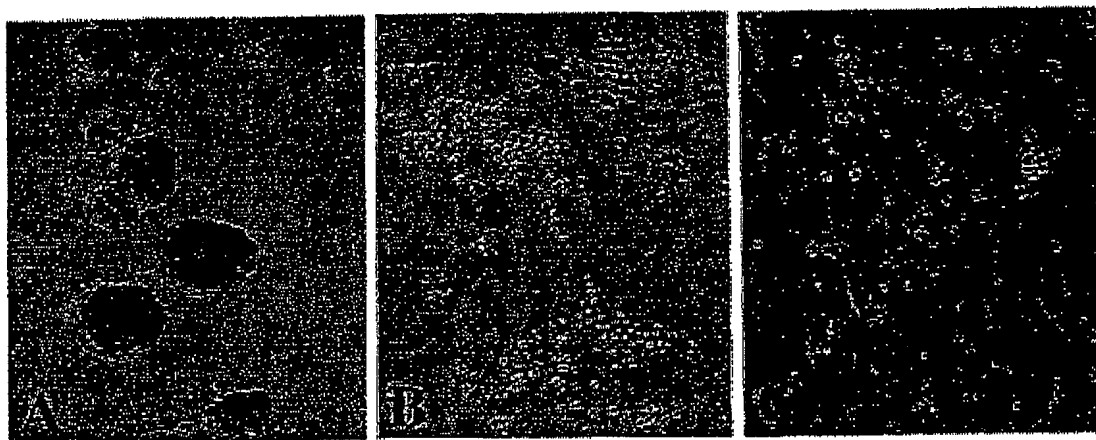


Fig. 6

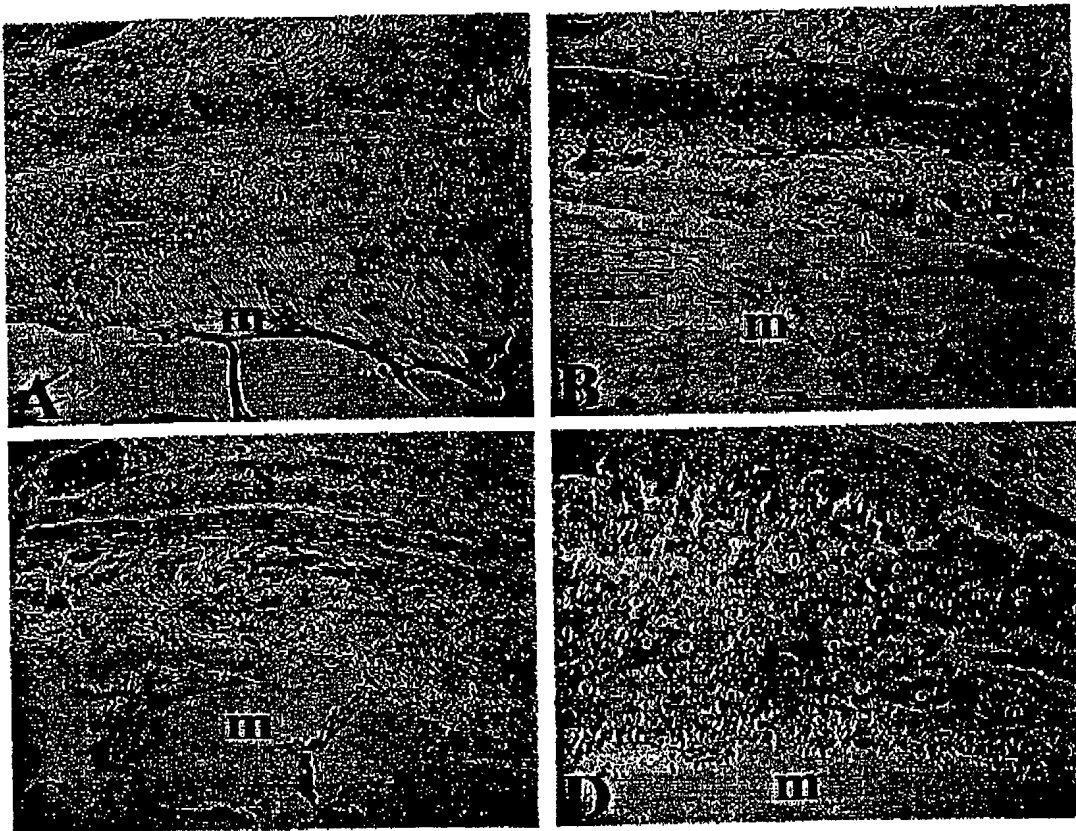


Fig. 7

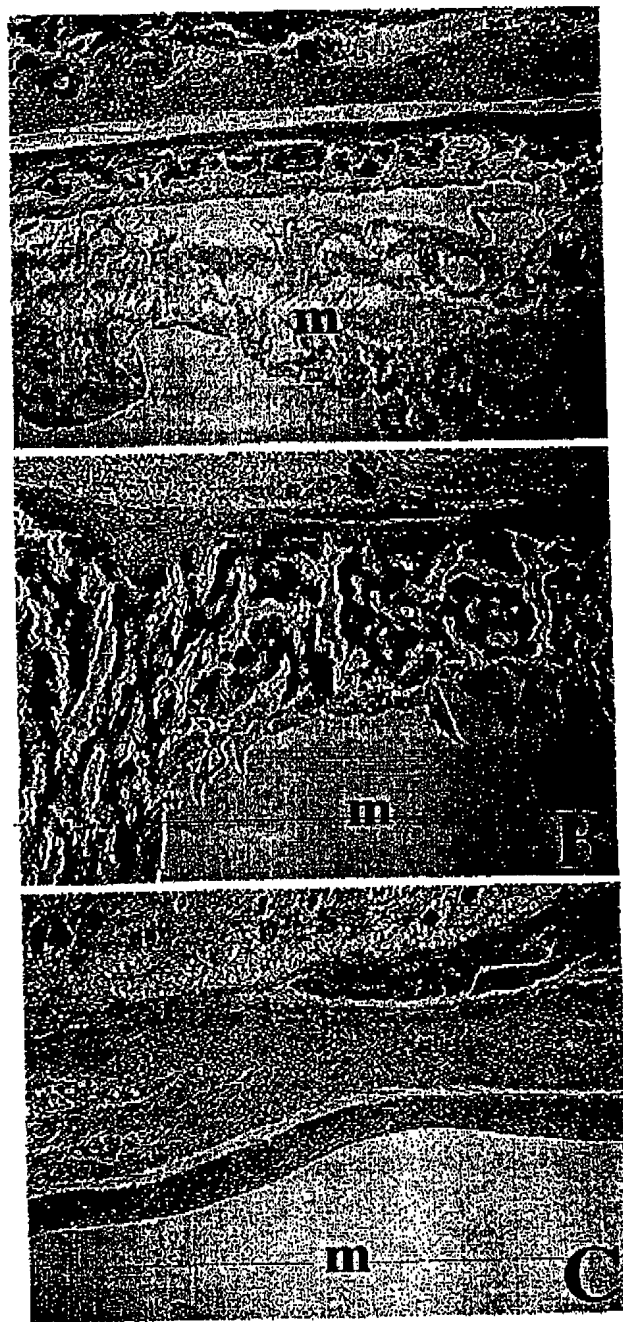
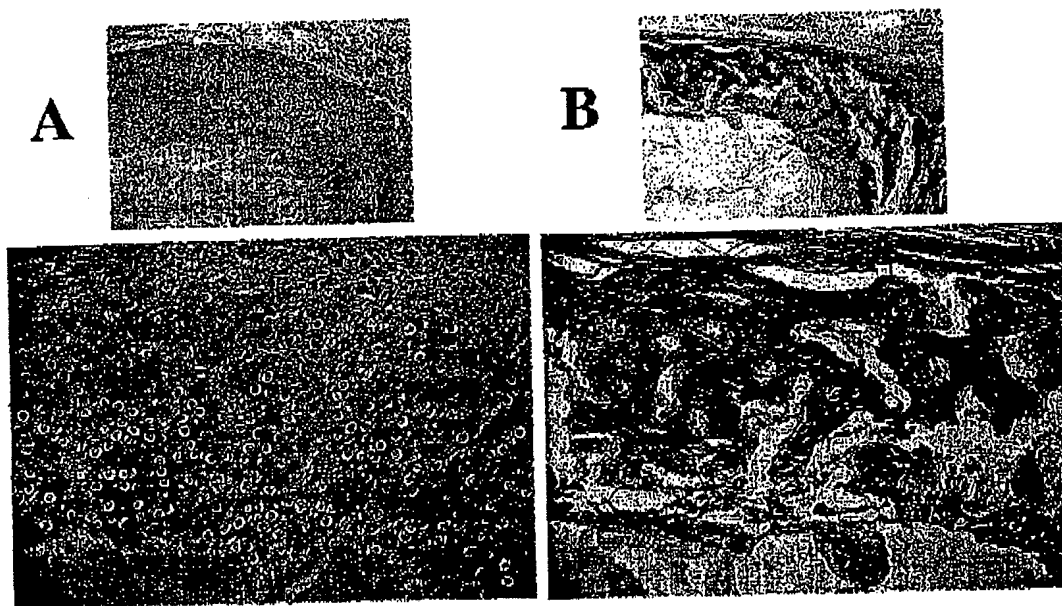


Fig. 8



INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 99/06913

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61L24/00 C08B37/08 A61L27/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C08B A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 24429 A (FIDIA ADVANCED BIOPOLYMERS S.R.L.) 14 September 1995 (1995-09-14)	1-3, 5-7, 10, 11, 13-20, 22, 23, 28-30, 33, 34, 39
Y	page 11, line 23; examples 1-11, 13-25	21, 24-27, 31, 32, 35-38
Y	page 21, line 30 - page 22, line 7	
	WO 97 45532 A (UNIV BROWN RES FOUND) 4 December 1997 (1997-12-04)	21, 24-27, 31, 32, 35-38
	page 7, line 18-30 page 13, line 1-8	
	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

Date of the actual completion of the international search

8 December 1999

Date of mailing of the international search report

17/12/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Radke, M

INTERNATIONAL SEARCH REPORT

Int ernational Application No
PCT/EP 99/06913

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 527 893 A (BURNS JAMES W ET AL) 18 June 1996 (1996-06-18) column 14, line 14-33; example 1	1,6,8, 11,13
A	US 5 652 347 A (POUYANI TARA ET AL) 29 July 1997 (1997-07-29) cited in the application column 3, line 6-16; claims 1-3,8; examples 1-5	
A	WO 97 18244 A (SEIKAGAKU KOGYO CO LTD ;WAKI MICHINORI (JP); MIYAMOTO KENJI (JP)) 22 May 1997 (1997-05-22) page 13, line 15-25 page 16, line 15,16; claims 1-5	
A	WO 90 06767 A (JOLLA CANCER RES FOUND) 28 June 1990 (1990-06-28) page 14, line 1 -page 15, line 4; claims 1,6; examples II,V	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 99/06913

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9524429	A	14-09-1995	IT PD940043 A	11-09-1995
			CA 2184899 A	14-08-1995
			EP 0749446 A	27-12-1996
			US 5856299 A	05-01-1999
WO 9745532	A	04-12-1997	EP 0907721 A	14-04-1999
			US 5939323 A	17-08-1999
US 5527893	A	18-06-1996	US 5017229 A	21-05-1991
			US 4937270 A	26-06-1990
			AU 670030 B	04-07-1996
			AU 2143492 A	30-12-1992
			AU 5226796 A	01-08-1996
			EP 0587715 A	23-03-1994
			JP 6508169 T	14-09-1994
			WO 9220349 A	26-11-1992
			US 5760200 A	02-06-1998
			AT 151294 T	15-04-1997
			AU 660282 B	22-06-1995
			AU 8392491 A	23-01-1992
			DE 69125609 D	15-05-1997
			DE 69125609 T	17-07-1997
			DK 537292 T	04-08-1997
			EP 0537292 A	21-04-1993
			ES 2100954 T	01-07-1997
			FI 925802 A	21-12-1992
			GR 3023436 T	29-08-1997
			NO 924875 A	16-12-1992
			WO 9200105 A	09-01-1992
			AT 138940 T	15-06-1996
			AU 606230 B	31-01-1991
			AU 2482588 A	17-04-1989
			CA 1332235 A	04-10-1994
			DE 3855351 D	11-07-1996
			DE 3855351 T	10-10-1996
			DK 68990 A	17-05-1990
			EP 0397652 A	22-11-1990
			FI 94357 B	15-05-1995
			JP 2670996 B	29-10-1997
			JP 9183804 A	15-07-1997
			JP 2684208 B	03-12-1997
			JP 3502704 T	20-06-1991
			NO 301770 B	08-12-1997
			NO 942763 A	16-03-1990
			WO 8902445 A	23-03-1989
US 5652347	A	29-07-1997	US 5616568 A	01-04-1997
			AU 1260295 A	19-06-1995
			WO 9515168 A	08-06-1995
			US 5874417 A	23-02-1999
WO 9718244	A	22-05-1997	AU 7587296 A	05-06-1997
			CA 2237192 A	22-05-1997
			CN 1207744 A	10-02-1999
			EP 0861270 A	02-09-1998
			NO 982212 A	14-07-1998
WO 9006767	A	28-06-1990	AT 135584 T	15-04-1996

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 99/06913

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9006767 A		AU 628910 B	24-09-1992
		AU 4828590 A	10-07-1990
		CA 2006060 A	20-06-1990
		DE 68926051 D	25-04-1996
		DE 68926051 T	29-08-1996
		DK 116691 A	17-06-1991
		EP 0449973 A	09-10-1991
		ES 2084688 T	16-05-1996
		NO 179505 B	15-07-1996
		US 5654267 A	05-08-1997
		US 5830504 A	03-11-1998
		US 5955578 A	21-09-1999